

Banking DNA sequences

MOUNT Everest, as we all know, was first climbed "because it was there". The Everest of those scientists who are devoted to the sequencing of DNA is the complete human genome, a peak that cannot yet be conquered. Nevertheless it seems not out of the question, even now, to attempt to sequence a single chromosome from man or *Drosophila*; or at least to tackle a substantial fragment of one such chromosome. And certainly some of the more ambitious sequencers would no longer consider the complete genome of the bacterium *Escherichia coli* to be a sufficient challenge to their skills.

The reasons why grandiose sequencing can now be contemplated are technical. In the early 1960s when Walter Fiers embarked upon the, then, highly ambitious project of sequencing the first complete genome, that of the bacterial virus MS2, it was not possible to sequence fragments of longer than eight nucleotides at one time. The complete structure of the 3,569 nucleotide genome of MS2 was published in 1976. Now, thanks largely to techniques developed by Alan Maxam and Walter Gilbert in Harvard and by Fred Sanger and his colleagues in Cambridge, one can sequence stretches of up to 250 nucleotides at a time. The sequencing of MS2 would now be no more than a single PhD project in a laboratory where the necessary techniques were already in operation.

Rapid sequencing, however, is only half the trick. One needs also relatively large amounts of pure DNA to be sequenced. For that, the techniques of cloning recombinant DNA in bacteria have been invaluable.

The combination of rapid sequencing and cloning has made it feasible to tackle larger and larger genomes. Following MS2, there came the bacterial virus ϕ X174 and the mammalian SV40, polyoma and hepatitis viruses. In the offing is the complete sequence of several more viruses, led by adenovirus, and that of the genome of mammalian and yeast mitochondria. In quantitative terms this is progress from the 3.6 kilobases (one thousand nucleotides) of MS2 to the 5.4 kilobases of ϕ X174, through the 35 kilobases of adenovirus to the 50 kilobases of yeast mitochondria.

By contrast the complete genome of *Escherichia coli* is about 10,000 kilobases, the *Drosophila* genome an order of magnitude greater and a single human chromosome some 500,000 kilobases of DNA. With a realistic outlook the *Escherichia coli* sequence could be completed by a team of ten in less than five years; with an optimistic one, a tenth of a human chromosome would not take as long because the very thought of devoting a lifetime to the task would force an order of magnitude increase in the speed of sequencing!

The dilemma for sequencers deciding what to tackle next is that, whereas a new graduate in the right laboratory might immediately stumble upon an iconoclastic stretch of DNA, so

might a bevy of experts take several years to reveal nothing more than another 1,000 kilobases of uninteresting sequence.

Not that it is possible to overestimate the astonishing discoveries that have emerged, unsuspected, from sequencing: overlapping genes, split genes, degenerate genes and the bizarre features of the mitochondrial genome, including a genetic code that breaks the rule that the code is universal.

As with so much in science, hunches may be the only way to make decisions, be they by project leaders or grant givers.

From any large scale sequencing there now arises a series of irritating problems. The first is in what quanta to report the sequence; gene by gene or only when the sequence of the whole genome is essentially complete? In theory the latter approach is preferable, unless any particularly interesting feature is revealed along the way. But can the pressure to milk a sequence for as many papers as possible, or even just one for each collaborator, be resisted?

Then there is the problem, faced by journals, of printing screeds of ATCG permutations, less appetising to those who have not acquired the taste, than even the stock exchange page of a newspaper. The temptation — perhaps to be resisted — is to photo-reduce them to near-illegibility. Worse only is the suggestion, sometimes voiced, that sequences should be available from the author, but not be published at all. That would serve the thinking reader about as well as does television's *The Forsyte Saga* act as a guide to Galsworthy's literary style.

Finally, one major problem arises from the quantity of sequencing that has already been amassed — of the order of 100 kilobases — let alone that which the next few years will bring. The problem is how best to collate as much data as possible so as to bring out salient features. Although number, or rather letter, crunching is no substitute for thought, nevertheless computers are an essential aid to sequence collation. Already they are needed in any large scale sequencing simply to piece together the data obtained from the sequencing of random fragments of large genetic units. What does not yet exist is any central data bank in which all sequences could be deposited and made freely available to the whole scientific community. There are good precedents for such a service in the Brookhaven Protein Data Bank and the Cambridge Crystallographic Data Centre. The need for a DNA sequence bank is becoming urgent. At its best it would operate on a computer network system and offer search programme facilities. At its most meagre it would be no more than a depository, run by a keyboard operator, which sent out tapes for a nominal fee. Proposals to start some kind of bank in the US have been in the air for at least a year, and last week the European Molecular Biology Laboratory veered towards a decision to start one. It matters little by whom, or where, a bank is started. What is important is that it should start soon. □



United States

More tests required on new chemicals

THE Environmental Protection Agency has for the first time used its powers to block the introduction of new chemicals, which have not been tested sufficiently for safety.

David Dickson reports

EVER since the Toxic Substances Control Act (TSCA) was passed by Congress in 1976, it has remained the centre of fierce controversy. The chemical industry has claimed that many of its rules on new and existing chemicals impose an unduly harsh economic burden; environmentalist groups argue that the Environmental Protection Agency has been dragging its feet in enforcing the Act.

Now the Agency has decided to bare its teeth. Under a section of the Act requiring all companies to provide pre-manufacturing notices (PMNs) containing data on safety tests before a new chemical is introduced, the EPA last week announced that it was blocking the manufacture of six chemicals for which it felt that data submitted were inadequate.

The name of the company is being withheld at its request. However the EPA said that the chemicals are six phthalate esters, members of a group of so-called plasticisers which are added to polyvinyl chloride products to make them flexible, and that proposed production levels would involve 300 to 400 production workers, and up to 10,000 plastic goods workers.

The EPA's actions are based partly on the results of a study being carried out by the National Cancer Institute which seems to be proving that DEHP, a widely-used plasticiser with a similar chemical structure, can cause liver cancer in mice and rats.

According to the agency, the company proposing to manufacture the new phthalate esters had not been aware of the NCI studies. And in submitting the PMN, it provided no reports on safety studies, leading the EPA to assume that none had been carried out.

EPA Deputy Administrator Barbara Blum said last week that, before manufacture is allowed to proceed, the company must provide information about the potential carcinogenicity of the new chemicals.

The action is the strongest so far to have been taken by the Agency under the TSCA. This reflects feeling among officials that some chemical companies are being excessively reluctant to provide data.

In a recent letter to a Congressional Subcommittee, for example, Dr Stephen Jellinek, Assistant Administrator for Toxic Substances, wrote that the lack of adequate test information was contrary to congressional intent in passing the Act, and "contradicts industry's oft-expressed view

of how it conducts its premanufacture activities".

Under the provisions of TSCA, companies must submit all existing test data about the safety of a new chemical, but are not required to carry out any additional tests unless the EPA determines that the production of the chemical could pose an "unreasonable risk" or would be in particularly large quantities. Environmental groups have criticised the flexibility of this arrangement. They argue that the EPA could have been more aggressive.

"The EPA says that the Act does not give them authority to promulgate rules for tests. But we feel that the Act does give it the power to gather any information they feel is necessary," says Dr Karim Ahmed of the Natural Resources Defense Council (NRDC).

The NRDC has already successfully sued the EPA over delays in implementing another section of the Act, which requires it to take action on a priority list of potentially dangerous chemicals drawn up by an interagency committee.

The Agency had argued that a 12-month time limit set by the Act required it merely to publish a progress report. However, three months ago a district judge in New York upheld the NRDC's contention that the actions themselves had to be carried out within this time. He said the 28 months which had elapsed since the priority list was published, and during which the EPA had taken no action against any of the listed chemicals, did not fulfil the congressional requirement.

NRDC members claim that the court victory strengthens their argument that the EPA could be moving more firmly to implement the provisions of the TSCA. In particular, it is arguing that the Agency has the power to set guidelines for the type of safety test data required to be submitted with PMNs.

This would bring the US more in line with actions recommended by the European Economic Commission that before starting production of a new chemical, a company would have to provide a certain amount of data on its potential environmental and health effects, in this case the amount of data depending on the proposed level of production.

The NRDC is proposing a 'tiered' scale of tests, with short term tests — for example for mutagenicity or chronic toxicity — required for all chemicals, and additional tests for those which are more

likely to be dangerous.

This approach, however, is being resisted by the chemical industry. "You have to consider how each chemical is going to be used. To have a laundry list of tests is too simplistic", says Dr Geraldine Cox, Technical Director of the Chemical Manufacturers' Association, who says that the current arrangement allows companies to talk to the EPA on a "scientist to scientist" basis.

Last week's actions indicate that the agency intends to "prosecute vigorously" the PMN provisions, according to one official. Its apparent commitment to a more aggressive stance on chemical testing was illustrated two days after the phthalate esters announcement, when Ms Blum told several leading chemical manufacturers that new safety tests would have to be carried out on the herbicide 2,4-D.

Unlike 2,4,5-T, 2,4-D does not contain dioxin contaminants, and there is no direct evidence that it is a health hazard. However, because of the structural similarity between the two substances, the EPA is demanding that new studies be carried out to eliminate any suspicion that it might be dangerous. □

Record rise in federal R&D backing

FEDERAL support for university-based research and development increased by \$610 million to a total of \$3,958 million between 1977 and 1978, the largest net growth since records were started in the early 1960s and the largest percentage growth since 1972, according to figures released by the National Science Foundation. The largest part of this increase came from the National Institutes of Health, whose support for university research increased by \$262 million. The 1978 figures represented an 8% real growth in funding over 1977.

The NSF statistics indicate that the majority of research support is concentrated in relatively few institutions. In 1963, when records were first compiled, 90% of federal R & D support went to the top 100 research universities. In 1978, the top 100 universities received 84% of all R & D funds, compared to only 56% of total federal funds to universities.

Top recipient was Johns Hopkins University, whose \$196 million of federal support — largely the result of incorporating for the first time the Defense Department-sponsored Applied Physics Laboratory — put the university far ahead of the previous leader, the Massachusetts Institute of Technology. □

A strong warning that, since the global build-up of atmospheric carbon dioxide will have varying climatic effects on different parts of the world, it could lead to increased tension between rich and poor nations, has come from a special committee of the Academy of Sciences.

In a report prepared for President Carter's Science Adviser, Dr Frank Press, the committee says that such a build-up will only be minimised by international agreement to reduce the use of fossil fuels and develop alternative energy sources. And in view of the potential divisiveness of the CO₂ issue, it recommends that "in the near term emphasis should be on research, with as low a political profile as possible."

The committee, which was chaired by Dr Thomas C Schelling, professor of political economy at Harvard University, was asked by Dr Press to look at the social and economic consequences of increased concentrations of atmospheric CO₂. Accepting the scientific consensus that such an increase is likely to take place, the committee concentrates on what it considers the most serious consequences, namely the relative distribution of the

CO₂ could increase global tensions

resultant gains and losses throughout the world.

It points out, for example, that developed countries with temperate climates are not only the largest consumers of fossil fuels, but may also benefit from the climatic effects, with raised temperatures and rates of photosynthesis leading to increased agricultural yields. Both the US and the USSR could benefit in this way.

In contrast, countries in subtropical arid zones could suffer a decline in rainfall and possible droughts with decreased food production and resultant shifts in population distribution — as well as potential demands for international compensation.

"It seems that climate change might well tend to make the already poor still poorer and increase the difference between North and South, rich and poor, developed and developing" says the committee.

Research on adaptive and preventive measures are, it says, "apparent and urgent" to help mitigate the consequences. For example it recommends efforts to improve the resilience of agriculture to climatic change. And in particular suggests that ways should begin to be explored of protecting low-lying land areas from the possible elevation of ocean levels due to the disintegration of the West Antarctic ice sheet.

Finally, responding to two recent reports in the scientific literature which suggest that the rate of carbon dioxide build-up may be an order of magnitude less than most scientists now fear — reports which have become the centre of fierce controversy in the climate research community — the committee says it agrees with the views of experts it consulted that "these are based on incomplete assessments that unrealistically omit important feedback processes." □

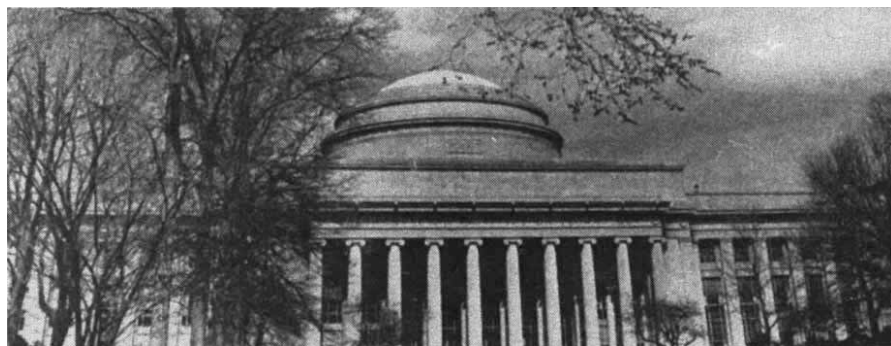
Exxon supports MIT research

BREAKING away from more conventional patterns of research funding, the Massachusetts Institute of Technology and Exxon Research and Engineering Company announced last week a ten-year agreement under which the company will provide up to \$8 million to support basic research at the institute into combustion processes.

In return, the company — the research arm of Exxon Corporation — will receive royalty-free, non-exclusive licenses to any patents which MIT obtains on technology arising from the research and will share any royalties on technology licensed to third parties.

Areas of research to be supported by Exxon include the burning of coal, coal liquids, shale oil and heavy crude petroleum. According to Dr Ed David, President of ER&E, a prime objective of the agreement is to "help generate the scientific base for more efficient and more environmentally acceptable burning of high sulphur, high nitrogen, hydrogen deficient fossil fuels" of particular interest to utilities and industrial companies.

Under the terms of the agreement, all results of the research can be published openly. ER&E will select the research projects it is prepared to finance from a list proposed by MIT. And in addition to this, an additional 20% of the total will be made



MIT: receiving industrial support for basic research

available by Exxon to MIT scientists to pursue their own combustion science research projects.

The two principal investigators at MIT will be Professor John P. Longwell, who joined the institute faculty in 1977 after a long research career with Exxon, and Professor Adel F. Sarofim. Initial research projects to be carried out under the agreement include studies of the relationship of flame hydrocarbon intermediates to surface deposits and hydrocarbon emissions; and the interaction of mineral matter with sulphur during coal combustion.

The agreement is, according to the university, "one of the largest and longest of its kind" and is the result of protracted negotiations between the two sides over terms. It cannot be terminated without two years notice from either side, and in the words of MIT president Dr Jerome Wiesner "provides the university with the certainty that these long-range research programmes will have the financial continuity and stability to ensure effective performance".

The novelty of the agreement lies in the

fact that whereas industrial support of applied research on university campuses is a common phenomenon, it is relatively unusual for a private company to sponsor more long-term, basic research.

This role has, at least in the post-war period, been conventionally assumed by the federal government, on the grounds that basic research is a common good. However, in recent months a number of industry executives — among which Ed David and other Exxon officials have been some of the more outspoken — have argued that the private sector should take on more responsibility for supporting 'goal-oriented' basic research carried out in universities, arguing that because of changing political, social and economic factors, government may not have the staying power or the style of management to meet industry's long-term needs.

Recently one Exxon official suggested that industry's share of support for basic university research should increase to 15% from its current level of about 3%. The MIT-Exxon agreement is one of the first steps in this direction.

David Dickson

Smallpox

Gone for good?

TODAY in Geneva, the World Health Organisation will officially declare smallpox eradicated from the Earth. It will be the culmination of an astonishingly successful 22-year programme by the WHO, which included, in the two years since the last endemic case was reported in Somalia, the unclaimed \$1,000 reward offered for new cases reported (*below*). But could smallpox ever recur? Peter Newmark examines the evidence.

THE WHO campaign to eradicate smallpox was launched in 1958, a year in which 250,000 cases of the disease were recorded. At first success was slow to come by. Even vaccination of 90% of the population of a country was sometimes found to leave a sufficient residuum of the virus for it to bounce back with a vengeance.

By 1967 the disease was still endemic, or in serious danger of being so, in 50 countries. The WHO therefore hatched and launched an intensified campaign of eradication.

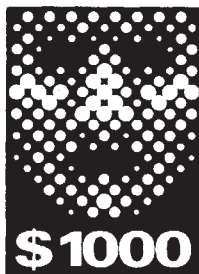
By any reckoning, the success of that campaign was remarkable. Ten years and \$300 million later the last case of endemic smallpox was recorded.

Two and a half years later, the WHO is convinced that smallpox has gone for ever. In coming to that conclusion the WHO has considered three possible routes of re-infection but deemed them to be most unlikely, if not impossible.

The first would be if the virus could lie dormant in smallpox scabs that have survived this long. But the WHO is satisfied that the virus quickly becomes harmless in scabs.

The second possible source of re-infection comes from the smallpox virus stocks that are being held in the seven WHO-approved and inspected laboratories. That possibility last became a reality in Birmingham, UK, in 1978 when one worker died and another became mildly ill. Since these were not endemic cases they did not count within the WHO's definition of the 'last' cases. They did however, lead the WHO to increase its biosafety requirements and inspections, and to speed up its policy of closing down as many smallpox laboratories as possible. In two years, it is hoped, there will be only four, but some will have to be maintained indefinitely because of the possibility, however remote, that the virus could reappear from some other source, necessitating a new programme of diagnosis and vaccination. WHO plans to keep in reserve sufficient freeze-dried vaccine for 200 million people.

Meanwhile research is continuing on the



Only five years ago . . . a WHO worker interviewing a child with smallpox in Ethiopia.

third conceivable route of reemergence, by mutation from monkeypox virus. That possibility was raised in 1978 by the publication in *Nature* of experiments carried out in the WHO-sponsored Research Institute of Virus Preparations in Moscow, under the direction of Dr SS Marennikova. These showed that both in the course of passage of monkeypox virus through hamsters, and in the pock appearing on chorioallantoic membranes infected with monkeypox, there sometimes emerged a virus that was indistinguishable from smallpox virus in laboratory tests.

That conclusion was controversial from the start because of the considerable differences between the smallpox and monkeypox virus; although both are members of the orthopoxvirus family, as is vaccinia virus from which smallpox vaccine is produced, monkeypox very rarely infects man and, when it does, appears not to be transmitted from person to person; in the laboratory every test, from its molecular characteristics to the colour of the pocks it produces, distinguishes it from smallpox. Whereas that does not preclude the possibility of mutation of monkeypox to smallpox virus, recent, as yet unpublished restriction enzyme analysis of the genome of twenty-one mutants newly derived from monkeypox show that, despite instances of major deletions and rearrangements of genes, all of the mutants were still recognizable as monkeypox virus, and none closely resembled smallpox virus. The laboratories responsible for this work (those of Prof. K R Dumbell, in St Mary's Hospital Medical School, London, and Dr J H Nakano at the Center for Disease Control, Atlanta, USA) have also analysed the genome of six of the mutants obtained from monkeypox in Moscow in 1978, and

the two monkeypox stocks from which they were derived. And at the molecular level, restriction enzyme maps of the two viruses show that the differences between them are so considerable that as Dr Nakano told *Nature*: "it seems highly improbable that the smallpox virus could ever be derived from the monkeypox virus by mutation".

However, Dr Nakano's laboratory has also analysed the four smallpox-like viruses isolated in Moscow from white pock on chorioallantoic membranes infected with monkeypox. Each of these mutants was indistinguishable from smallpox virus, confirming Dr Marennikova's identification of them.

The current position therefore seems to be that whereas it has not been possible to reproduce Dr. Marennikova's derivation of smallpox virus from monkeypox virus, and the genomic differences between the two viruses make such an event highly improbable, yet the evidence is clear that the Russian mutants are indistinguishable from smallpox.

There are two possible explanations, both unpalatable in their own way. The first is that Dr. Marennikova's results were the result of contamination. Obviously that can never be ruled out, and one of her two original monkeypox virus strains does appear to show signs of contamination with smallpox virus; however, the other does not.

The other possible explanation, and much the more worrying one, is that, despite the improbability, monkeypox virus *can* mutate to smallpox virus. That possibility is described by WHO as "highly unlikely, but impossible to disprove". All it can do is to continue to sponsor research — and hope that today's declaration of smallpox eradication is the very last one. □

Cell culture

Fetal calf serum drought hits cell culture laboratories

CELL culture laboratories throughout the world have been hit by their own fuel crisis — a shortage of fetal calf serum, the magic component of the media in which many biologists grow their cells. Supply has become uncertain, and the price has escalated; in the UK it has doubled in the past year and is now pushing £100 a litre.

Scientists are now exploring alternative ways of growing cells. At the very least they are in for a lot of trial and error, and time consuming work, as cells are weaned off fetal calf serum. Some projects will have to be abandoned.

Changes in world agricultural policies and practices lie behind the shortage, says Mr Tom Coutts, the managing director of Gibco Europe Ltd., which with Flow Laboratories, is the main distributor of fetal calf serum in Europe. He believes the overall supply will be bad for one or two years but may ease in the long term.

Flow was unable to supply any fetal calf serum at the end of April, and the company described the shortage as "very, very serious". In late April Gibco wrote to customers saying "severe shortages" would affect supply "for the foreseeable future . . . it is in our customers' interest to take every possible step to reduce their dependence on this product". Both companies hope the short term situation will be boosted by the start in April of the serum collection season in Australia and New Zealand, Europe's main sources of supply.

Fetal calf serum is a by-product of the beef industry. It is extracted by cardiac puncture when a cow brought to market for slaughter is found to be pregnant. Mr Coutts traces the current shortage back three years when beef prices were low, cattle were slaughtered rather than raised for the meat market, and calves, which would have become today's breeding stock, were lost. Now beef prices have risen and calves are being reared. Also, the US has lifted beef import quotas against Australia, making it even more profitable for Australian farmers to keep their calves.

Fetal calf serum is widely used by research institutes, hospitals, and universities for medical research, and by pharmaceutical companies in drug production. Its application includes use in diagnostic procedures, cancer research, and virology.

In the past the serum has been freely available, with just the occasional minor problem of supply. It has also been relatively inexpensive and extremely effective, curiously for reasons not yet understood. Its widespread introduction

during the 1960s meant an end to the practice of scientists undergoing a self-imposed 24-hour starvation and obtaining a pint of their own blood for use in the medium.

Dr John Birch of Searle Research and Development says that "a great mythology" surrounds fetal calf serum. Dr Eric Hall, an English scientist at Columbia University in New York, says: "Tissue culture work using fetal calf serum has got a bit out of hand. We have sloshed it around because it was cheap and available. Some work has been done just for fun. We need to rethink our priorities." Now many laboratories will have to stop using it.

The Imperial Cancer Research Fund (ICRF) in London, which has about 70 people in tissue culture work, replenishes its supplies monthly and is now receiving about half the fetal calf serum (75 litres a month) it needs to meet its planned research programme. ICRF scientists have been asked to halve the amount of serum they use in media, to 5%.

University departments simply do not have the money to keep paying for the serum. Small laboratories are faced with a dilemma: either they pay high prices with the knowledge that they can successfully grow their cells, or they gamble with cheaper sera which may not give the desired results.

Professor Sydney Shall of the Biochemistry Department at Sussex University suggests that the number of places able to do tissue culture work will decrease. "It's a grave issue," he says. His cell lines are sensitive human cultures such as brain cells and they need 10% fetal calf serum. His department is rethinking its research programme.

Dr Colin Arlett of the Medical Research Council's Cell Mutation Unit, at Sussex, can provide an example. He is growing fibroblasts from patients suffering from ataxia telangiectasia, a genetic disease. These cells are difficult to grow even on fetal calf serum and "if it becomes completely unavailable, or the price prohibitive, work on these cells will have to cease."

Cell biologists in Europe are no longer given the luxury of testing batches of fetal calf serum before purchase. They have been told by distributors to take what they are given or look for another source. Without testing, there is no guarantee that a particular batch of serum will support adequate growth of the cells being used.

"At least we have a buffer compared with smaller laboratories," Mr William House, manager of research services at

ICRF, said. "If a particular batch of fetal calf serum doesn't suit one person's cell lines, it can be used by someone else."

The attitudes and methods of biologists themselves may be contributing to the problem. Panic buying has occurred over the last 12 months says Mr Coutts. The demand is artificially high, fed by the rumours of the impending short supply. Unwillingness to try alternatives and over dependence on fetal calf serum may also be factors.

In searching for alternatives, the most popular solution is likely to be weaning the cells onto other sera. Indeed, some scientists claim that they predicted problems of supply of fetal calf serum and have successfully changed to other sera. Alternative sera are less costly and more readily available. However, there may also be some problems.

New born calf serum, aseptic calf serum, and horse serum will support the growth of some cells, although usually with poorer growth rates; and some primary cell lines may have an absolute requirement for fetal calf serum.

Another complicating factor is whether or not the properties of the cells will change. Once the cells are growing on other sera, partially completed projects face the possibility of a change in the cellular response to the treatment — such as irradiation or drugs — under investigation. Earlier experiments which used fetal calf serum may have to be repeated. Dr Hall has already reported such cellular changes in response to chemotherapeutic drugs.

Many scientists believe that the ideal solution to the dual problems of the supply of serum and the variability it causes in the growth of cells can be solved by the use of a totally defined or synthetic medium. Attempts over the last 20 years to identify the magic ingredient(s) in fetal calf serum have not been successful; the latest shortage may revive this work. Several cell lines already grow on a totally defined medium and the popularity of these cells is sure to increase.

A further question arising from the shortage is the effect it will have on the antivivisectionist movement which believes that biologists should turn to tissue culture in preference to research using animals. Legislation to encourage this development has been proposed in the UK and elsewhere in Europe. But biologists working with animals are unlikely to change to tissue culture if they see such problems with the supply of serum.

Ian Anderson

Ian Anderson is a freelance journalist

NEWS IN BRIEF

EEC funds 10 new solar energy projects

THE Commission of the European Communities approved last week grants to 10 new solar energy projects totalling 3 million EUA (1 EUA = £.665). The projects include an award of 53,000 EUA to K.U. Leuven of Belgium for an electric car using solar energy and nine awards to solar heating projects which include block central heating by solar power for 250 houses in Alpes-Maritime in France, solar heating of greenhouses in Italy, a West German project to study solar heating of swimming pools and a project for solar energy heating of phosphorising vats at a refrigerator production plant in Pordenone, Italy.

UK locates geothermal energy source

AN exploratory borehole at Marchwood, near Southampton has located a large deposit of water at a temperature of 70°C the Secretary of State announced in Parliament last week. The water was found at a depth of 5,600 feet and can be brought to the surface at a temperature of 65°C a drop of only 5°C. There is sufficient water in the well to heat about 1000 houses over a period of several decades. Pumps will be installed late this summer to bring the water to the surface and its mineral content is being assessed.

Geothermal energy has been in use in Hungary since the beginning of the 20th century and has found increasing uses in France to heat apartment blocks in the Paris suburbs. France plans to have half a million geothermally heated buildings by 1990 which will produce a 15% saving in running costs. The UK's £1.8 million

borehole project is part of a £11 million yearly programme to explore alternative energy sources including wave, wind, tidal and solar energy.

UK Seveso report issued

AN English translation of the 1978 Italian Parliamentary Commission report on the Seveso accident was published by the UK Health and Safety Executive last week. The Executive clearly feels that the Seveso accident involving the discharge from a chemical factory of some 250-500 grams of 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (dioxin) over a populated area was serious enough to warrant a report of the causes and consequences of the incident being made more widely available. But central to the concern over the health of the Seveso residents was the two week delay in ordering their evacuation from the dioxin-contaminated area: on this point the report has been criticised as inaccurate. For a detailed discussion see *Nature* 22 February 1979, page 588.

Seveso: A translation by the Health and Safety Executive of the official report of the (Italian) Parliamentary Commission of Enquiry. £25.00

Queen's Award to new, safe insecticide

A GROUP of UK scientists received a Queen's Award last week for their development of a class of highly active insecticides that have low mammalian toxicity. Chemists and biologists at the Rothamsted Experimental Station solved the problem of making synthetic pyrethroid compounds, known from the group's previous research to attack insect but not mammalian nervous systems, stable against exposure to sunlight. According to Paul Needham of the Rothamsted Station the compounds breakdown rapidly in soil and residues are unlikely to accumulate to contaminate the environment. Three compounds of the new class of insecticides, permethrin, cypermethrin, and decamethrin, are sufficiently active to be used at dosages of an order of magnitude less (10-200 g/ha) than that of most other insecticides (500-1500 g/ha). The pyrethroids are effective against lepidopterous larvae in cotton, timber, wool and stored products.

Collection of Yeast Cultures finds home

THE UK National Collection of Yeast Cultures, threatened by withdrawal of funds by two of its sponsors last January (*Nature*, 24 January page 325) will be moved to the Agricultural Research Council's Food Research Institute in Norwich before the end of the year. The Collection, consisting of 1,500 yeast strains widely

used throughout Europe was saved by a decision of the Advisory Board for the Research Councils to provide the money to move and maintain the collection. The ABRC will provide about £24,000 annually so that the Collection can carry out its central functions of maintaining and supplying fully characterised yeast cultures to industry, universities and schools as well as acting as a reference and information centre for identification and deposit of yeast cultures. The collection will continue to be housed in its present quarters, the Brewery Research Foundation, until the transfer is effected during the summer.

Research could help industry: Canadian report

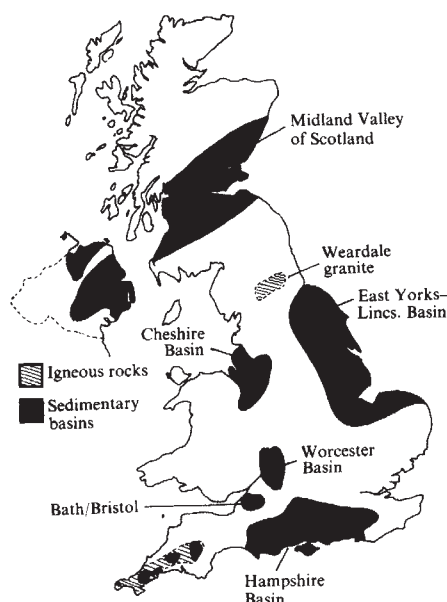
INCREASED support for basic research in areas such as materials science and processing, vehicle control systems and battery electrochemistry could provide a long-term boost to the Canadian car industry, according to a report prepared by Arthur D Little of Canada Ltd for the Ministry of State for Science and Technology.

The report, prepared on parallel lines to an initiative (already under way) of the US government to stimulate research through projects jointly supported by federal and industry funding, lists a number of areas of "directed basic research opportunities" relevant to automotive technology which might be performed in universities or research institutes. "This kind of research is not directly intended to result in the design of new vehicles, engines, or components; it is meant to focus on achieving a fuller understanding of the physical and chemical processes underlying vehicle technology," the report says. But it adds that programmes of this nature, with a time horizon for commercialisation primarily in the 10 to 20 year range, must receive adequate government funding in order to be successful. "There are a large number of government sponsored research programmes in Canadian universities and government research centres that we believe are underfunded," the report says.

Errata

In the leader of 17 April, page 583 David Howell was erroneously referred to as the UK opposition spokesman on energy. This should have been David Owen.

The letter from John Goddier in 24 April, page 658 was written in his capacity as Branch secretary of the Agricultural Research Council Branch Institution of Professional Civil Servants. It does not represent an official ARC view.



A birthday party with real rockets

Bulgaria is celebrating 1,300 years of nationhood next year, and scientific events are among the festivities. But as **Vera Rich** reports, the best birthday present the country could have would be a pollution-free water supply

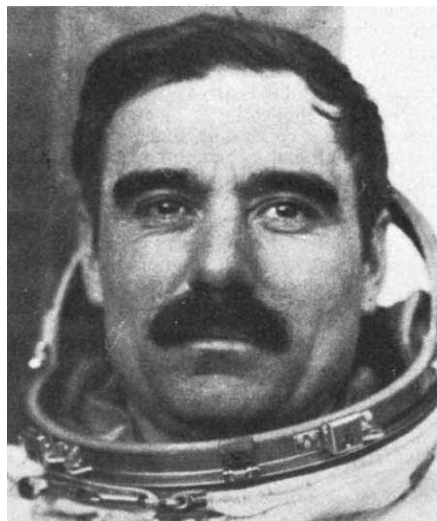
LATER this month, Bulgaria will hold its second national conference on molecular biology — the first scientific event of the jubilee.

Dedicating a scientific congress to an event in the remote past may seem novel to western eyes, but it is merely one of a whole series of scientific events associated with the anniversary. In spite of its relatively small size and low science budget (about 1.3% of the GNP) Bulgaria is doing work at an international level in a number of fields, molecular biology included.

According to Dr Rumen Tsanev, of the Institute of Molecular Biology of the Bulgarian Academy of Sciences, work in molecular biology began some 10 years ago — before the Soviet decrees of 1974 which assigned priority to catching up in molecular biology and genetics. Lysenkoism, however, never really took hold in Bulgaria; there was some theoretical discussion of it in the 1950s but this was quickly over and, said Professor Tsanev, there was no persecution of people who did not accept it.

Bulgarian work on molecular biology has included: the discovery that the nucleosome consists of two sub-units; the formulation of mathematical models of cell differentiation; study of the role of histones in DNA transcription; and, most recently, two-dimensional electrophoresis studies of differences in the primary structure of nucleosomes. As for genetic engineering, "We feel it can only be developed as part of general molecular biology", Tsanev explained. "We started only last year, and at present we are simply trying to introduce methods of handling and mastering the techniques". Preparing too for genetic engineering, is Dr Basil Staikov of Bulgaria's unique "Rose Institute" at Kazanlak, a research station devoted to every aspect (from biochemistry to the mechanics of harvesting) of the Kazanlak roses whose essential oils play so significant a role in the Bulgarian economy. For the moment, however, Dr Staikov explained, Kazanlak has not the equipment for genetic engineering, and new varieties are still obtained by traditional cross-breeding methods; γ -irradiation experiments begun some four years ago, said Staikov, have so far given no definite results, although the general indications seem "promising".

Bulgaria is extremely proud of the scientific traditions it has established during the last century or so. The Academy of Sciences was founded in 1869. Bulgarian



Citizen-cosmonaut Ivanov. His space mission failed last year — but the scientific side of the programme was carried through.

scientists have been particularly active in solid state physics; but last year, according to Dr Milko Borisov, Director of the Academy's Joint Centre for Physics, the best results in Bulgarian physics came from work on liquid crystals, dielectric and flexielectric properties. Specialists from the Centre are cooperating with Polish and Italian teams on an "Atlas of liquid crystals" to be published by the Bulgarian Academy, probably in 1981.

International projects, naturally, cannot be dedicated to the Bulgarian jubilee. Nor, it seems, can the new astronomical observatory at Roehen, with its 200cm hyperbolic mirror with a Richey-Chretien-Coudé focus system. Much of the optical equipment came from Karl Zeiss (JENA) and when the observatory is formally opened later this year, it seems likely that it will be dedicated to DDR-Bulgarian friendship.

However, Bulgarian statehood will be celebrated in astrophysics. A special satellite Bulgaria-1300 will be launched by a Soviet rocket towards the end of the year. The experiments it will carry are, for the moment, a secret, but, according to Dr Kiril Serafimov, head of space research, Bulgaria is an active participant in all five sections of the "Interkosmos" programme — space physics, meteorology, communications, space biology/medicine, and remote geophysical sensing. Even before "Interkosmos" was set up as the official organisation for Comecon joint research, Bulgarian physicists assisted their Soviet colleagues in evaluating data from

the "Elektron" probe — and later, from US satellites.

The Bulgarian public is still hopeful that their "space" celebrations might include the launch of a second Bulgarian cosmonaut, and with greater success than Georgi Ivanov, who with a Soviet crew-mate, failed last year to link up with Salyut-6. But 20 of his scheduled experiments were carried out by the Soviet team on board including work with the Bulgarian Spektr-15 remote sensing camera (which weighs only 15.2kg but has 15 channels), the casting of an aluminium-hydrogen foam, work on epitaxial surfaces and crystal growth, and electrophotomagnetic experiments on the aurora. Only the seven medical experiments scheduled for Ivanov had to be abandoned.

Perhaps the biggest scientific contribution to the jubilee will be the publication of Bulgaria's "Red Book" of endangered species. According to Professor Simeon Nedyalkov, of the Institute for the Coordination of Research for Environmental Conservation Bulgaria is, from a geobotanical point of view, a "melange" of boreal, mediterranean, pontic and Asia-minor species. Since the "technical revolution", especially around the country's new metallurgical plants, many species are threatened. There has been over-cutting of valuable timber; snowdrops have been depleted by the demands of the medical profession (they yield nivalin — an alkaloid used in the treatment of polio); and a well-intentioned attempt to step up trout production by introducing American varieties proved disastrous, for the newcomers failed to adapt to local plankton and became predators on the native trout.

Now, however, said Professor Nedyalkov, Bulgaria has a well established national monitoring system for all forms of air and water pollution, and in especially menaced areas, soil pollution also; and work is going forward on the resistance of plants to toxic elements.

Bulgaria's concern for the environment extends to the international level — not surprisingly, since its only sizeable river, the Danube, arrives too polluted even for irrigation. The existing Danube Commission covers only pollution from ships, and Bulgaria has been one of the most active of the riparian states in pressing for a Danube Convention to cover all aspects of monitoring and research into water quality and ecology. The preliminary text for a Convention has already been drafted and, said Dr Veselin Petrov of the Committee of Environmental Protection of the Council of Ministers, it is hoped that a final text will be signed this year. That would be the best 1300th birthday present Bulgaria could receive from the up-stream Danube countries. □

CORRESPONDENCE

Tumour surveillance in beige mice

SIR, — Since beige mice have been shown to have defective natural killer (NK) cells¹, several groups have reported recently in *Nature* on the presumed consequences — viz. poor immuno-surveillance for spontaneous tumours in mice^{3,4} and men⁴. Padgett⁵ reviewing Chediak-Higashi-syndrome of man, mink and cattle noted raised incidence of lymphoma. Beige mice are the murine equivalent. The gene, *bg*¹, was long ago obtained from the Jackson Laboratory on its C57 BL background and in this laboratory crossed onto a (C₃H × 101)F₁ background and maintained thus. Neither of these Harwell strains nor the F₁ is noted for natural lymphoma. After the reported NK defect in C57 BL *bg bg*, 40 Harwell beige mice were set aside for life-time study. Others had been used for determining the origin of the osteoclast from the haematopoietic stem cell^{6,7} during which electron microscopy studies of their bones had revealed C type virus, often in substantial numbers, in young mice. Of the 40 mice, now aged 15-18 months, 11 (27%) have died or been killed with a variety of disseminated lymphomas. The remainder suffer from ataxia noted around the age of one year and currently under investigation.

Yours faithfully,

J.F. LOUTIT
K.M.S. TOWNSEND
J.F. KNOWLES

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Oxon., UK

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What should be called a lectin?

SIR, — During the past decade, interest in lectins (also referred to as agglutinins, phytohemagglutinins, phytoagglutinins and protectins) has increased enormously. The term lectin has frequently been used to describe substances which differ markedly from those classically considered lectins, that is plant seed carbohydrate binding proteins. Nevertheless, the discovery of lectin-like substances in widely diverse sources (bacteria, fungi, fish sera, snails, etc) appears to warrant an expansion of the term. For the sake of clarity we would suggest the following definition of "lectin".

A lectin¹ (from the Latin *legere*: to chose) is a sugar-binding protein or glycoprotein of non-immune origin which agglutinates cells and/or precipitates glycoconjugates.

● Lectins bear at least two sugar-binding sites, agglutinate animal and plant cells (most commonly erythrocytes, unmodified or enzyme-treated) and/or precipitate polysaccharides, glycoproteins and glycolipids.

● The specificity of a lectin is usually defined in terms of the mono-saccharide(s) or simple oligosaccharides that inhibit lectin-induced agglutination (or precipitation or aggregation) reactions.

● Although first discovered in plants, lectins also have been found in many organisms from bacteria to mammals. A lectin may be soluble in biological fluids (eg concanavalin A) or membrane-bound (eg rabbit or rat liver lectins).

● There are several other types of sugar-binding proteins including sugar specific enzymes (glycosidases, glycosyltransferases, glycosylkinases, glycosylpermeases, glycosylphosphatases . . .), transport proteins, hormones (thyroid-stimulating hormone, follicle-stimulating hormone, . . .) and toxins (ricin, abrin, modeccin . . .), etc.

Under some conditions, sugar-specific enzymes with multiple combining sites agglutinate cells (and/or precipitate glycoconjugates) and so act as a lectin.

In spite of similarities to true lectins from the same source, toxins — which bear only one sugar-binding site — should not be called lectins since they do not agglutinate cells or precipitate glycoconjugates.

Yours faithfully,

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NATHAN SHARON
The Weizmann Institute of Sciences,
Rehovot, Israel

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Thought for lowly locusts

SIR, — We were surprised, nay disappointed by the conclusion to your article (24 April, page 659) entitled 'Pores for thought', namely "There is every reason to believe . . . and will be followed by those of channels induced by a variety of neurotransmitters". May we remind you that *Nature* published a paper last year entitled 'Single glutamate activated channels on locust muscle' (278, 643; 1979). Are we to believe that glutamate is not a serious contender as a transmitter? Perhaps the lowly status of locusts precludes them from serious consideration by Acting Editors of eminent journals.

Yours faithfully,

P.N.R. USHERWOOD K.A.F. GRATION
The University of Nottingham,
UK

Amanda and the scientists: a verse on syntax

SIR, — The following verses are intended to both amuse and instruct your readers, many of whom, judged by their contributions to your pages, are guilty of faults of syntax:

"Papa," said Amanda, "I want to know what These scientists mean when they write:
'The dye was identified using a plot
Of E against wavelength of light.'"

"Or here: 'The bacilli were easily seen
When using high magnification

And judging by leucocytes found in the spleen
They had caused an acute inflammation.'"

"And in *Astronom. Rev.* I read: 'Bearing in mind
The value ascribed to log g
Circinus X-1 is now better defined
As a neutron low-mass binary.'"

"But can germs use a microscope, dyestuffs a plot?"

Are bacilli with judgement endowed?
Is it logs on the mind that makes neutron stars hot?

I protest that my mind's in a cloud."

Said Papa, "Your confusion is natural enough
But in time you will find it amusing
To know you can always spot scientists' stuff
By this typical misuse of using."

"Misattached participles are constantly seen
In scientists' journals today
For scientists rarely can say what they mean
Or, in consequence, mean what they say."

"They treat with derision linguistic precision,
Their syntactical errors are massive
And chiefly because of the quaint superstition
That the Voice of true Science is passive."

"Still, think of the blessings by Science
bestowed:

The quasars and antibiotics;
The strontium-90, the helical code,
Carcinogens, quarks and narcotics!"

"Oh I do!" said Amanda, "Yet since you've explained
That their writings need mental translation
To be understood, I conclude that, though
trained,

Most scientists lack education."

"That remark," said Papa, "though quite
frequently heard
No iota of difference will make:
They language and logic abuse undeterred
With a confidence nothing can shake."

"To a study of leptons or nebulous Crabs
Ne'ertheless you may one day aspire.
For a start go and visit the Veterinary labs
And admire what there is to admire."

"Yes I will!" said Amanda, and seizing her coat
Ran off to that innocent band
Who were busy improving Rebecca the goat
By transplanting her mammary gland."

Yours faithfully,

R.F. GLASCOCK
With acknowledgements
to Belloc.

Reading, UK

Taxi illusion — explained

SIR, — Surely the explanation of the "taxi illusion" (24 April, page 658) is as follows:—

Suppose the taxi is travelling at speed *V*. The passenger, looking back through the rear windscreen sees objects along the road receding at speed *V* relative to the taxi.

Looking forward, he sees the reflected images of those objects, apparently moving forward at speed *V* relative to the taxi.

He sees the images against a background of real objects seen through the front windscreen moving towards the taxi at speed *V*.

The reflected images therefore appear to be passing the real objects at a speed 2*V*.

Yours faithfully,

J.P. FREEMAN

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NEWS AND VIEWS

Icebergs: technology for the future

from Herbert E. Huppert

EVERY year some 5000 icebergs, totalling 10^{12} m³ of ice, are calved from Antarctic glaciers and ice shelves. Each iceberg consists of approximately 10^8 tons of pure fresh water. It drifts for a number of years under the influence of the currents and the winds, gradually breaking up and melting into the Southern Ocean. Occasionally, icebergs drift as far as 30°S, and may influence regional climate as well as local weather. At the time of calving each iceberg has a flat top and vertical sides which plunge some 200m into the water. After several years they may be worn into very convoluted shapes as can be seen in the cover illustration.

A recent conference on The Use of Icebergs* was mainly devoted to discussions of the physical characteristics of icebergs, including the way they break and roll over, their melt-rate, and the feasibility of capturing icebergs and towing them to Australia, South Africa, California or Saudi Arabia, where they could be used as a supply of fresh water.

Icebergs break due to the flexural strains induced by ocean swell and differential melting. Breaking can be enormously enhanced by the presence of flaws such as the cracks and deep crevasses that are frequently found in ice shelves. P. Wadhams (Cambridge University) presented calculations for the elastic response of a perfect, two-dimensional ice sheet to a given swell-induced pressure distribution at the base of the ice. The results yielded the maximum strain as a function of the amplitude of swell and size of the ice, the ice fracturing when the strain exceeds a postulated breaking strain. His calculations suggest that large ice sheets will not be broken up in seas which can destroy smaller sheets. Only very large icebergs would thus be expected to remain intact as they drift into regions far from the Antarctic coast. This fits well with the

observations that few medium-sized icebergs are seen in these areas. O. Orheim (Norsk Polarinstitutt, Oslo) reported that the 1978/9 Norwegian Antarctic Research Expedition found very few crevasses at the base of the 24 tabular icebergs on which they landed. If this is generally true, it suggests that icebergs are calved from the ice shelf along every basal crevasse, although it is possible that the ice around each crevasse had melted since calving.

The processes of iceberg melting were surveyed by H. E. Huppert (Cambridge University), who argued that the base of an iceberg was likely to melt less than the vertical sides. Melting at the sides of an iceberg into an ocean of uniform density leads to a turbulent, entraining plume containing upwardly moving melt water mixed with oceanic salty water. In regions where the ocean is vertically stratified, the melt water largely flows out in a horizontal series of layers (Huppert & Turner, *Nature*, 271, 46; 1978 and *J. Fluid Mech.*, in press). There was evidence of both forms of convection in measurements taken around a grounded iceberg in Conception Bay, Newfoundland by E. G. Josberger (Oregon State University). An experiment in which a block of ice $1\text{m} \times 0.5\text{m} \times 0.25\text{m}$ was melted into water at approximately 20°C and 35‰ was described by D. S. Russell-Head (University of Melbourne). Under these conditions it appeared that the basal melt rate was comparable to that for the vertical walls. This is possibly due to melt water formed at the base being able to flow up the sides.

Calculations for the frequencies of oscillation of an iceberg of given shape, and the shapes which lead to instability and hence roll-over, were the subject of a number of presentations. J. F. Nye (University of Bristol) presented an analysis based on catastrophe theory. From Nye's results it can be inferred that if the melt-rate increases with increasing depth the iceberg remains stable and is unlikely to roll over. This melting pattern was noted in the experiments above but in the Antarctic the melt-rate may be sufficiently influenced by local variations

in temperature and current speed to alter the resultant geometrical shape. All the calculations neglected the induced motion in the water, although this has been found by naval architects to be important (J. N. Newman, *Marine Hydrodynamics*, M.I.T. Press).

A number of results are already available from the 1978/9 Norwegian Antarctic Research Expedition. This was one of the first expeditions to obtain field data from Antarctic icebergs rather than just ice shelves. South of about 66°S, the measured annual melting was less than a few centimeters. Measurements of the temperature of the upper 10m of the bergs indicated that the temperature rose above that of the -20°C of the ice shelf to -5°C and higher. The expedition employed a sidescanning sonar to determine the underwater shapes of icebergs and ice shelves. The most exciting result, reported by B. A. Fossum (Continental Shelf Institute, Trondheim), is the discovery of an underwater ram of ice protruding more than 180m at a depth of 100m from the ice wall at Kapp Norvegia. This contrasts with the completely vertical face mapped at Blåenga which is less than 100km away.

Iceberg Transport International have carried out a number of scaled towing tests and some of the results were reported by F. Mauviel (Iceberg Transport International, Paris). The tests consisted of towing a wooden 1/100 scaled model of a tabular iceberg in Brienz Lake, Switzerland, and a steel 1/60 model in St. Malo Harbour, France. Propulsion was provided by outboard motors, or by sails, or by hauling on lines attached to a very large parachute drogue ahead of the model. The last means of propulsion appears to be the most efficient. Extrapolating from the test results, Mauviel estimated that a reasonable efficiency could be achieved by using two drogues of 10^5 m² to propel an

*The conference, organized by The International Glaciological Society, was held at the Scott Polar Research Institute, Cambridge, UK from 1-3 April, 1980 and was sponsored by Iceberg Transport International, King Faisal Foundation and Abdul-Aziz University through the initiative of Prince Mohammed al Faisal al Saud. The proceedings are to appear in *Annals of Glaciology*, 1.

Herbert E. Huppert is in the Department of Applied Mathematics and Theoretical Physics, University of Cambridge.

iceberg through the water at a speed of 0.5 knots. The two drogues would be pulled alternately, operating, in principle, like the hands of a swimmer doing the Australian crawl. The drag acting on the models was noted to be a fairly strong function of shape and to be least for an "iceberg" whose maximum girth is amidships and whose vertical walls are tapered so that the bow and stern width are approximately 3/5 of the central width. It is envisaged that the next series of tests will involve an actual Arctic iceberg that is roughly 1/10 the size of an Antarctic berg.

A study by D. J. DeMarle (Rochester Institute of Technology, New York) focused on the feasibility of processing icebergs in Saldanha Bay, on the west coast of South Africa. The region has been listed as a future metropolitan growth area by the South African Department of

Environmental Planning. Instead of melting the iceberg on arrival, DeMarle proposed that it be mined using existing technology. A slurry of ice fragments and water would then be pumped ashore via an ocean pipeline. The operation might be powered by the waste heat of a nearby industrial complex, for which a large cold sink could help to increase efficiency.

M. W. Holdgate (Department of the Environment, UK) highlighted the audacious simplicity and social benefits of using icebergs as an inexhaustible source of water. However, such a scheme is not without dangers, not only because of accidents which could result from a stream of broken off icebergs being abandoned in shipping lanes but also because we have little idea of the environmental effects of moving icebergs around the world. □

SRS-A and the leukotrienes

from W. Dawson

THE discovery of a new class of biologically active compounds is always exciting, even at the present time when knowledge is advancing so rapidly.

The leukotrienes are a new structural class deriving from arachidonic acid, a precursor which has already given us the prostaglandins, thromboxanes, prostacyclin and various hydroxy fatty acids. The systems from which the leukotrienes have been released and characterized are closely related to immediate hyper-sensitivity reactions and it has been proposed that SRS-A, slow reacting substance of anaphylaxis is a member of the leukotriene family.

Slow reacting substances (SRS) are so-called because of the characteristic slow contractile response they induce in smooth muscle preparations *in vitro*. The original SRS from lung tissue was described by Kellaway and Trethewie in 1940, being released from guinea pig lung by snake venom. In a series of papers in the early 1950's Brocklehurst isolated and characterized a similar substance released from guinea pig lung following anaphylactic challenge and named it SRS-A. He later identified a similar product released *in vitro* from human lung, derived from an asthmatic person in response to the specific allergen. This work clearly linked SRS-A with the long lasting bronchoconstriction experienced by asthmatics.

For the next 25 years many research groups attempted to identify the chemical structure of SRS-A, notably those of Brocklehurst himself in England and Austen with the late Robert Orange in the USA. Although advances were made, the structure remained elusive. Undoubtedly the extreme potency of SRS-A, the relatively small amounts of material available, and its unusual physico-chemical properties were the major causing slow progress of this research.

During the 1960's and early 1970's the prostaglandin (PG) family of structures were identified and their pharmacology described. It seemed natural that the analytical challenge of SRS-A should attract workers in the PG field and within a relatively short period of time publications from many research groups appeared in the literature. The independent work of Parker, Piper and Bach all suggested incorporation of radiolabelled arachidonate into SRS-A-like biological activity whilst two groups, at St. Louis and Lilly, showed incorporation of 35 sulphur. The Harvard group identified sulphur by spark emission spectroscopy in their SRS-A preparation and it seemed clear that it was only a matter of time before the structure was revealed.

During 1978 and early 1979, both Parker and Jakschik in St. Louis and Piper and Morris in London suggested various functional groupings which they felt were present in the molecule. In particular, the chromophore at 278-280 nm seemed a key to the purification of the minute amounts

of available SRS, so described because the bulk of the research over this period concentrated on material chemically released with the ionophore A23189, rather than the immunologically generated SRS-A.

In May 1979, at the international prostaglandin conference in Washington, Bengt Samuelsson of the Karakinska Institute in Stockholm, in collaboration with Borgeat, Hammarstrom and Murphy described the work which led to the characterization of the leukotriene (LT) structures LTA, LTB and LTC from a murine mastocytoma cell line and from rabbit polymorpho-nuclear leukocytes. LTA was shown to be 5.6 epoxyarachidonic acid, LTB a dihydroxy-arachidonic acid and LTC as 5 hydroxy 6 cysteinyl arachidonic acid. His suggested name derived from the leucocytes which release the material and the conjugated triene system of double bonds in the structure. These double bonds were also responsible for the chromophore at around 280 nm, an observation made by both Piper's and Parker's groups.

A relationship was suggested between this chemically released SRS, now to be known as leukotriene C, and SRS-A but the picture was not yet complete. At the end of 1979, and in early 1980 Samuelsson, in collaboration with the chemical group of Corey at Harvard, described LTC as glutathionyl hydroxy arachidonate. Comparison of the biological activities of the natural with totally synthesized material showed them to be identical. It should be stressed, however, that this natural material was chemically released and so could not be correctly called SRS-A. Also, early in 1980, Piper and Morris characterized an SRS chemically released from guinea pig lung as glycy-cysteinyl hydroxy arachidonate, differing from the LTC described by Corey and Samuelsson by a glutamic acid residue.

In this issue of *Nature* (p. 104) Piper and Morris describe the first characterization of immunologically released SRS-A from guinea pig lung, achieved with a novel mass spectrometric procedure, and show that it is identical with the chemically released material released from this tissue.

Developments in this field are so rapid, however, that Samuelsson described the same compound at the Winter Prostaglandin Conference in Snowbird, Utah this April and showed that it was formed from leukotriene C by the enzyme A-glutamyltranspeptidase. He named it leukotriene D and described, in collaboration with a number of his colleagues, various aspects of the pharmacology of the leukotriene compounds: A, B, C and D.

It is pertinent to note that the majority of the data presented by the various groups is chemical in nature and the biological activity of these compounds will need to be clearly defined. No doubt in the ensuing months biological flesh will be put on these exciting chemical bones. □

Is the homing pigeon's map geomagnetic?

from Bruce R. Moore

RACING pigeons released six hundred miles from home often return within the day. Their remarkable performance reflects true navigation, not piloting or random search. The animals are known to obtain directional information from the sun, and appear to possess, in addition, a magnetic compass. Although the precise mechanism of magnetic detection remains unknown, two possible receptors have now been identified (see Presti & Pettigrew, this issue of *Nature*, p. 99). Moreover, there is evidence that the earth's field may provide not merely directional, but also positional, information: a compass, but also a map.

The pigeon's use of a solar compass has been shown in several ways but most dramatically in a demonstration in which the birds were made to fly, essentially, north, south, east or west by phase-shifting their diurnal rhythms (Schmidt-Koenig, *Cold Spr. Hrbr. Symp. Quant. Biol.* **25**, 389; 1960). Normal animals treat the direction of the noon sun as south and, when released, "home" properly. If, however, their diurnal rhythms have been advanced by six hours, they treat the sun's noon azimuth as west, and if retarded by six hours, as east; such animals fly 90° left or right of home. Others, phase-shifted by a full 12 hours, err by 180°. In each case the birds are seen to correct for the sun's apparent movement at an average rate near 15°/hour.

"Clock-shift" experiments such as these work, and should work, only when the sun is visible. When the sky is heavily overcast, experienced birds are still able to home, whatever the phase of their rhythms (Keeton, *Science* **165**, 922; 1969). Obviously, then, there is another mechanism besides the solar compass. This alternative appears to be magnetic.

The seminal paper on magnetic effects was that of Keeton (*Proc. natn. Acad. Sci. U.S.A.* **68**, 102; 1971) which showed that fastening strong magnets to the backs of experienced birds interfered with their orientation on sunless days. Three years later, Keeton *et al.* (*J. comp. Physiol. A.* **95**, 95; 1974) found that pigeons' bearings were affected by even minor fluctuations in the geomagnetic field. Changes of less than one milligauss — a five-hundredth of the natural field — were accompanied by appreciable anti-clockwise deflections of the birds' initial flight bearings. This was confirmed by Larkin and Keeton (*J. comp. Physiol. A.* **110**, 227; 1976); and again by Richardson (*Ibis* **118**, 309; 1976) in observation of diurnal migrants. Again with migrants, Wiltshko and Wiltshko (*Science* **176**, 62; 1972) appeared to show that the magnetic receptor, whatever and wherever it might be, reacted not simply to

the horizontal component of the earth's field but, rather, to its direction of inclination; Walcott and Green (*Science* **184**, 180; 1974) reported consonant findings with pigeons.

The nature and location of the magnetic receptor remained a mystery, but the literature provided several clues: the experiments of Keeton and of Walcott and Green suggested that the organ should first be sought in the animals' heads or necks; additionally, it was known that organisms as diverse as bacteria (Blakemore, *Science* **190**, 377; 1975), molluscs (Lowenstam, *Science* **156**, 1373; 1967) and arthropods (Gould *et al.*, *Science* **201**, 1026; 1978) are able to synthesize particles of lodestone — or, more properly, magnetite.

The same substance has now been detected in the heads and necks of homing pigeons (Walcott *et al.*, *Science* **205**, 1027; 1979; Presti & Pettigrew, this issue of *Nature*, p. 99).

Walcott *et al.* analysed more than twenty pigeon specimens and found, in each, a single small pocket of magnetic material, just beneath the skull. Electron-probe and X-ray analysis, colour, and a Curie point near 580°C, collectively revealed the material as Fe₃O₄, magnetite. Electron-microscopic analysis showed numerous small elongated particles, of a size and shape which would act as elemental magnets, i.e., as single domains (Butler & Banerjee, *J. geophys. Res.* **80**, 4049; 1975). The tissue contained perhaps 10⁷ such particles. It also contained nerve fibres, although of unspecified origin.

Presti and Pettigrew did not encounter this structure in any of nineteen pigeon specimens which they examined. The reasons for the discrepancy are not entirely clear, but it should be noted that both groups of investigators were obliged to work with cumbersome glass dissecting tools, and that, perhaps as a consequence of this, the anatomic location of the structure had not been specified.

Presti and Pettigrew did, however, discover other deposits of magnetite. These were in neck muscle and surrounding tissue in both homing and feral pigeons, and various species of migrants. The authors suggest a functional connection with sensitive stretch detectors.

The physical basis of the suggested mechanism is quite straightforward. Magnetite is composed of ferrous (FeO) and ferric (Fe₂O₃) oxides, and thus contains three Fe ions. The electron spin of one of

these is characteristically opposite to the others, giving a net magnetic effect. Magnetic poles exist in pairs, and in the Earth's weak, locally-uniform field, dipoles twist toward alignment. Whether the resultant torque could be detected, even by spindle receptors, in the *Sturm-und-Drang* of flight might perhaps be debated. But, as with the subcranial structures of Walcott *et al.*, the ultimate test of the proposed receptor will be electrophysiological.

When the avian magnetic receptor is finally and unequivocally demonstrated, a fundamental functional question may remain. Does the receptor supply a compass alone, or also, perhaps, a "map"? This possibility was touched upon by a number of speakers at a recent symposium*.

The Earth's field contains positional, as well as directional, information. Between the magnetic pole and equator, the field's strength and inclination, and therefore the strengths of its horizontal and vertical components, change systematically. Any two of these measures, or any one of them plus declination, could be used to form a bico-ordinate map — an imperfect map in many ways, marred by anomalies and ambiguities, and subject to distortion by magnetic storms, but nonetheless, perhaps, serviceable (Talkington, *A.A.A.S.*, 1964).

In fact, birds released near magnetic anomalies do behave as if their "maps" have been affected (Walcott in *Animal migration, navigation, and homing*, Springer-Verlag, 1978). The related effects of magnetic storms have been known for decades (Thauzies, *Arch. de Psychol.* **9**, 66; 1910), but have not received sufficient attention. The most informative data here are those of Keeton *et al.* (op. cit.) showing anti-clockwise deflection of pigeons' initial flight bearings. Storms of apparent magnitude (K_{FR}) 3-5 caused deflections of 13° at one release site and 36° at another. One might suppose that the birds' compasses had been deflected, but field rotations in magnetic storms do not reach 36°, or 13°, or even 1°. The effect on the birds was clearly magnetic (see Larkin & Keeton, *J. comp. physiol. A.* **110**, 227; 1976), but could not have involved any obvious sort of compass.

A more plausible explanation would be that the storms had somehow affected the animals' sense of location. One release site was north and slightly west of the home loft, the other west and slightly south. In each case the animals reoriented as if they had been transported several tens of kilometers southwest of their true locations. At the times of release, and throughout the

*A symposium on navigation by homing and migrating birds sponsored by the Rockefeller University was held at Millbrook, New York from April 11-12th, 1980. A.J. Lednor and C. Walcott of the State University of New York, Stony Brook; J. Gould and J. Kirschvink of Princeton and B. Moore of Dalhousie University, Halifax mentioned the geomagnetic "map" hypothesis.

preceding nights, the magnetic storms had changed local field strengths by, at most, a few parts per thousand. The birds' sensitivity to these small changes was astonishing. The scale factors cannot be determined precisely from published data, but were of the order of 10° – 40° rotation per milligauss. The birds reoriented as if the storms had produced magnetic fields (or

perceived fields) like those normally prevailing southwest of the actual release sites.

Many other "maps" have been proposed, based upon solar position or movement, stars, sounds, odours, coriolis forces, landmarks and inertial guidance. The data have not, to date, supported even the most plausible of these hypotheses. I think that, perhaps, the time has come to take seriously an earlier suggestion (Viguer, *Rev. Phil.* 14, 1; 1882) that the pigeon's map is geomagnetic. □

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Superfluorescence: macroscopic quantum fluctuations in the time domain

from Q.H.F. Vreken, M.F.H. Schuurmans and D. Polder

THE process of spontaneous emission of radiation from a collection of excited atoms is commonly called superfluorescence. Superfluorescence produces radiation pulses which have much larger amplitudes than those which one would obtain in normal incoherent atomic radiation processes. The phenomenon is intrinsically quantum mechanical because the decay of the entire excited system is initiated by a single spontaneous emission due to quantum fluctuations.

Spontaneous emission by excited atoms has been commonly viewed as a random process in which the stored energy is released in the natural life time τ_n of the excited state. However, in 1954 Dicke¹⁾ predicted that under certain conditions the energy is released cooperatively and in a much shorter time $\tau_R \propto \tau_n/N$, where N is the number of excited atoms. Correspondingly, the emission intensity is proportional to N^2 instead of N as expected for random individual emission. Of course, such an N^2 intensity behavior is well-known from the coherent emission of driven electric dipoles. However, excited atoms have no dipole moment; classically the atoms would not even radiate. Nevertheless, the prediction is that cooperative behaviour develops in this system and leads to an emission intensity proportional to N^2 and an emission time proportional to τ_R/N — the phenomenon of superfluorescence (SF).

The first experimental observation of SF was made by Feld and collaborators²⁾ in 1973. Since then SF has been observed on a number of near-infrared, infrared and optical transitions (Ref. 3 and references therein). SF from a pencil shaped volume of active atoms has been studied systematically³⁾ in atomic cesium. The SF emission from the pencil goes mainly

through its end-faces into two narrow solid angles $\Delta\Omega$. The emitted light pulse reaches a peak intensity proportional to N^2 at the so-called delay time $\tau_D \propto (\tau_R/4) (\ln N)^2$ where $\tau_R \propto \tau_n/(N\Delta\Omega/4\pi)$. The emitted field behaves as a classical coherent field. It has a well-defined amplitude and phase: SF emission pulses from two different samples with slightly different SF transition frequencies produce beats³⁾. When the atomic system is repeatedly raised to the same excited state by single shot pulse excitation, the emitted SF pulses show macroscopic fluctuations from shot to shot in properties such as delay time and shape. These fluctuations are of quantum-mechanical origin. Such experiments provide a unique opportunity to study macroscopic quantum fluctuations in the time domain. This phenomenon has recently attracted the attention of theorists and experimentalists³⁻⁹⁾.

The fluctuation behavior observed here may be compared with that of a needle initially placed upright in the gravitational field. In a gaseous atmosphere atoms will collide with the needle, providing a stochastic force which turns the needle away from its upright position. With increasing polar angle θ , gravity will contribute more and more and eventually the trajectory of the needle becomes deterministic with a fixed azimuthal angle ϕ . When the needle is repeatedly put upright its trajectory is unpredictable as regards ϕ . The time it takes the needle to reach the horizontal position fluctuates from experiment to experiment. The fate of the needle is mainly determined by the unpredictable events in the early stage of the experiment.

The unpredictability of the path of the needle from one experiment to the other results from the incomplete knowledge of the system as regards the positions and velocities of the colliding atoms. In quantum mechanics a different kind of

incomplete knowledge arises. It is related to the Heisenberg uncertainty in the results of measurements on two non-commuting observables. Often this uncertainty may be accounted for classically by introducing fluctuations in the various quantities. SF is initiated by such quantum fluctuations. The initially excited atoms are in an energy eigenstate. The energy does not commute with the electric dipole moment and consequently the dipole moment is uncertain. The electromagnetic field is initially in the vacuum state, with no photons present, and in that state the electric field is uncertain. The initiation of SF requires a quantum-mechanical description. Early important contributions of this nature are due to Bonifacio and Lugiato⁴⁾. Recently, Glauber and Haake⁵⁾ (GH) and the present authors⁶⁾ (PSV) have obtained analytical solutions for the early stage of SF evolution by linearizing the equations of motion and decoupling the forward and backward emission. In the GH work the SF starts from the quantum fluctuations describing the uncertainty in the initial dipole moment; such a description is particularly suited to the calculation of the electric field variables. In the PSV work the SF starts from the quantum fluctuations of the electro-magnetic field in vacuum; such a description is particularly useful for the calculation of the atomic variables. The two approaches have also been combined in one complete linear quantum theory⁷⁾. Haake and collaborators⁸⁾ have extended the linear theory into the non-linear regime by assuming that the system evolution becomes already classical in the linear regime. They have numerically calculated single shot outputs.

In the above theories the various fluctuating quantities are expanded in plane wave modes passing through the pencil in all directions. The dominant gain of the modes in the solid angles $\Delta\Omega$ is used to describe the formation of two collective plane-wave-like dipole modes along the pencil axis. This collective, antenna type, behavior in the forward and the backward direction leads to the N^2 peak emission intensity and the characteristic emission time $\tau_R = \tau_n/(N\Delta\Omega/4\pi)$.

The collective behavior of the atomic system is very similar to the behavior of the needle mentioned earlier. Different single shot SF outputs correspond to different trajectories of the needle after it has been put upright. The eventually deterministic behavior of the needle corresponds to the classical behavior of the SF single shot emission field; collective field and dipole modes eventually outgrow the noise in strength. The SF pulse has a fixed but unpredictable phase and its delay time fluctuates from shot to shot.

SF is particularly interesting because the quantum fluctuations reveal themselves in the time domain. The strength of the initiating quantum noise determines the average delay time. The stochastic properties are manifest in the delay time

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fluctuations. Experiment⁹ confirms the prediction of the linear theory⁵⁻⁷ that the strength of the initiating quantum noise is such that, on average, SF is set off by the first photon emitted spontaneously in the solid angle $\Delta\Omega$. The relative standard deviation in the delay time of 12% at $N = 10^8$ atoms, as predicted by both the linear and the more complete nonlinear⁸ theory, is in good agreement with preliminary measurements by one of us³.

It is amazing indeed that such large fluctuations, quantum mechanical in origin, can be observed in nearly classical coherent pulses containing as many as 10^8 photons. □

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Sun-weather effects

from R. Gareth Williams

IN a recent paper, Nastrom and Belmont (*J. geophys. Res.*, **85** C1, 443; 1980) have found an interesting correlation between the 11-year sunspot cycle and the upper troposphere and lower stratosphere. They have thoroughly and carefully analysed data from the Northern Hemisphere for a period covering 1949 to 1973, i.e., just over two solar cycles. Their results indicate that during the winters of this period, the average position and, to a lesser extent, the strength of the jet stream and the intensity of features such as the Siberian upper level trough, have a strong correlation with solar activity. It is not so much the statistical significance of the results which is surprising, but rather that in certain areas of the hemisphere 50% of the interannual variance is explained.

In order to consider the significance of these new findings, it is necessary first to review the general state of sun-weather effects. Despite almost 180 years of study, we have little evidence to support the hypothesis that solar activity significantly affects the troposphere. There is a plethora of statistical correlations, but many of these have been produced by over-filtering and selecting the data and the remainder have nearly all failed where the data sets have been extended in time. Also, there are no thoroughly evaluated mechanisms which explain how solar activity may affect



100 years ago

A Parisian speculator has inaugurated the aeronautical season by a private ascent on April 25 at La Villette gasworks. The balloon, of only 300 cubic meters capacity, bore one aéronaut, with 30 kilograms of handbills, which were distributed all over Paris. The wind being slight, with a favourable direction, thousands of these prospectuses were picked up by street passengers and largely read. The whole expense of the aerial expedition, gas and everything, did not exceed 10*l.* sterling.

The French Minister of Fine Arts has entered into an agreement with the Jablochhoff Electric Light Company to light the palace during the whole of the two months devoted to the exhibition. The number of lights fed by the machinery is about 400, and the motive power

regarded at about 320 horses. The inauguration was to take place on May 1, and a large crowd had congregated to witness the process. But the crank of one of the principal engines broke, and it was necessary to postpone the opening for a few days. In spite of the growing opposition of the friends of the gas company, M. Garnier, the architect of the Paris Opera, will establish a trial of the principal electrical burners, to decide which is the more really fit for use in the house.

We learn from Catania, under date April 26, that the inhabitants were apprehending an eruption of Etna. An immense cloud of smoke has been observed.

A contemporary gives the following method of illustrating the indestructibility of matter: — Two sealed glass tubes of equal weight, one of them containing oxygen and a little powdered charcoal, are prepared. The charcoal may be caused to burn away completely by heating it by means of a small flame. On placing the two tubes on a balance it will be seen that there has been no variation in weight. from *Nature* **22**; May 6, 17&18; 1880.

either the climate or day-to-day weather. Indeed, there is a good reason why it is difficult to establish such a causal link; the energies associated with solar activity are very small when compared with tropospheric values (see, for example, Willis, *J. Atmos. Terr. Phys.*, **40**, 513; 1974). This means that any proposed mechanism will necessarily be indirect and complicated, and also that any statistical correlations, on their own, must be viewed with caution.

So, with this particular study by Nastrom and Belmont, although there is no doubt that the correlations are real (question marks raised by the authors over spatial coverage and the number of independent stations should only alter the details of the conclusions) the crucial, unanswered question is: 'Are the correlations caused by the changes in solar activity?'. The authors suggest that their findings may eventually be explained in terms of a direct solar effect on the stratosphere/mesosphere which, in turn, could modulate the upward propagation of tropospheric energy. There is clear theoretical and experimental evidence for the August 4th, 1972 solar proton event affecting stratospheric ozone. However, relationships between the solar cycle and ozone are somewhat controversial and thought at best to be small ($< 1\%$) (Dutsch *J. Atmos. Terr. Phys.* **41**, 771; 1979). Correlations between other stratospheric parameters and the solar cycle are similarly not yet fully accepted and are generally based on short time series. The authors refer to the atmospheric model of Bates (*Quart. J. R. Met. Soc.*, **103**, 397; 1977)

which had a troposphere that was very responsive to changes in the stratosphere. More recent work by Shutts (*Quart. J. R. Met. Soc.*, **104**, 331; 1978) shows that this sensitivity is model dependent. Both studies use linearised equations. Nastrom and Belmont cannot indicate which aspect of the solar output is thought to be causing their correlations, so we do not know where in the atmosphere any proposed mechanism should start.

If we jump the gun (by quite probably at least a decade) and assume that these correlations are solar induced, well understood physically and that these upper troposphere effects manifest themselves at the surface, then it is appropriate to consider what their impact on climate forecasting might be. Since 11-year trends in seasonal averages have been analysed, there will be no effect on day-to-day weather forecasts, nor on the 'long range' forecasts produced a month or so in advance. However, in localised regions of the globe it might become possible, for example, to forecast a run of 2 or 3 years with, on the average, cold winters. Such forecasts could, of course, have enormous economic impact. The drawback is that it is also necessary to be able to predict solar activity!

In conclusion, it seems unlikely that this present study will, on its own, alter the opinions of most meteorologists who view sun-weather effects with extreme scepticism. Only some very solid physics will do that and much theoretical work, coupled with further data analysis, will be needed to provide such firm footing. Even though it is free of the criticisms levelled at so many previous studies in this field, this paper will undoubtedly suffer, rightly or wrongly, from so many past correlations which were later found to be invalid. □

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The emergence of Man

from Glyn Isaac

ON March 12th the Royal Society and the British Academy convened a two day meeting to consider "The Emergence of Man". The central problem, presented by the chairman J. Z. Young, was to identify the kinds and timing of changes that led to human characteristics and the evolutionary pressures that produced them. The conference was characterized by the diversity of the methods being used to tackle these problems, contributions coming not only from paleontologists and anatomists but also from biochemists, immunologists, archaeologists and social psychologists.

Garniss Curtis (University of California, Berkeley) summarised geochronological information on the dates of important anatomical changes. In particular, evidence from fossils and footprints now demonstrates that bipedal locomotion was adopted between 3 and 4 million years ago. The age of the oldest fossils that show significant brain enlargement and which are classified into a species of the genus *Homo* has been the subject of controversy. A large series of new potassium-argon and fission track dates now all show that Koobi Fora early *Homo* specimens, including skull 1470, are close in age to the Olduvai Bed I *Homo habilis* fossils, that is between 1.7 and 2.0 million years in age. (*Nature* 283: 368; 1980).

Elwin Simons (Duke University) reviewed information from fossils of the forerunners of bipedal hominids. Formerly the *Ramapithecus* specimens from the Siwaliks and from Kenya have seemed the most likely fossil ancestors of man. Simons showed that while this is still possible a spate of discoveries of fossils of *Ramapithecus*-like creatures in different parts of Eurasia complicates interpretations. These new finds from Greece, Hungary, Turkey and China indicate wider geographic range and more diversity than had been expected. Further study and the recovery of more associated postcranial material is needed before the phylogenetic relationships of the various forms can be regarded as settled. The oldest securely dated, substantial sample of early hominid skeletal remains are the 3 to 3½ million year old fossils from the Afar in Ethiopia. Russel Tuttle (University of Chicago) reported briefly on features of the Afar hand and foot bones. He indicated that in spite of numerous primitive and ape-like characteristics there was evidence of

anatomical shifts in the human direction. He was confident that the hands could have made and used tools and he showed that even ape hands have considerable ability in this regard.

J. Lowenstein (University of California, San Francisco) reported on some entirely new research which could have far reaching effects in the testing of phylogenetic hypotheses. He succeeded in extracting small samples of collagen, albumin and transferrin from fossils, and using the techniques of radio-immuno-assay he has succeeded in measuring immunological differences between the fossil proteins and living organisms. A series of tests, including on forms such as fossil mammoths, fossil bison and various marsupials have indicated the taxonomic reliability of the method. Clearly, when determinations have been made for *Ramapithecus* the results could help to settle the long-standing controversy over the date of divergence between apes and hominids.

The meeting circled rather warily round its central challenge — the evolution of the human brain and its relation to human abilities. R. E. Passingham (Oxford University) reported on new experimental work that explored the function of Broca's area. It seems likely that the area does not control sound production itself but rather the way in which sounds are assembled into sequences. Chimpanzees may have no need for such an area because their natural calls are not made up by varying the sequential order of elementary units. It was suggested that the development of such centres, where intricate sequences of motor control impulses are sent to a central organ, is accompanied by the development of dominance by one of the cerebral hemispheres. The localisation of motor control over human language in one hemisphere is paralleled by similar neural mechanisms in song birds and Passingham predicted that lateralisation might also be found in 'singing' cetaceans. The reason for lateralisation may be simply to avoid interference from two controlling centres.

Ralph Holloway (Columbia University, New York) reported on the development of new techniques for establishing quantitative models of the shapes of human and hominid cranial endocasts. Not only have several hominid taxa turned out to be distinguishable from each other but some brain regions where differences are strongly represented; notably the lower parietal lobule, the anterior occipital zone and the dorsoanterior region of the frontal lobe, have been identified. The integration of this kind of research with information on brain function is clearly of importance.

Tool making and archaeology were also explored as sources of evidence regarding developments in brain function. Tobias pointed out that the transition to bipedal locomotion and the acquisition of enhanced manipulatory abilities occurred long before the fossil record gave any indications of major changes in brain form

and size. Previously he had advocated tools as the crucial innovation, now he suggests instead that about 2 million years ago a situation arose in which biological and cultural evolution were in a positive reciprocal feedback relationship. In this "autocatalytic system", speech, along with tools, was a crucial component.

A somewhat different view was presented by Glyn Isaac (University of California, Berkeley). He suggested that the early representatives of the genus *Homo*, who between 1-1/2 to 2 million years ago formed the oldest known archaeological sites at the Omo, Olduvai, Koobi Fora and elsewhere, were non-taking, non-human hominids. The formation at that time of clusters of artefacts and of broken up bones does, however, encourage the hypothesis that the hominids had a social system involving food-sharing. This system could in turn have created novel selection pressures towards the development of language.

Further archaeological evidence was reported by Peter Jones (Oxford University). In experiments at Olduvai he made stone tools similar to those found at various Acheulian sites, in each case using the correct local raw material. The raw material has a strong influence on the finished form of the tool; this may well raise problems for our understanding of the development of tool making.

A new approach which may allow us to determine the diet and nutrition of early man was described by Alan Walker (John Hopkins University, Baltimore). Scanning electron microscopy of the occlusal surfaces of cheek teeth allows us, at least, to clearly state what early man was not eating. The early hominid species were not feeding either on grass (including grass seed heads) nor were they crunching bones in their jaws. The wear and polish found on early hominid teeth is comparable to that found on primates and other forms which feed on fruits and soft leaves. Biochemical studies also suggest that the robust australopithecines were not eating foods that required a different pattern of mastication from that of the gracile forms but simply had to process more food per jaw movement.

A dramatic find was described by Mary Leakey. A trail of footprints discovered at Laetoli in Tanzania has been shown to date to between 3-1/2 and 4 million years ago. Leakey believes that the trail belongs to a phase when human ancestors had become upright bipeds with their hands free of locomotor function but when involvement in stone tool making had not yet begun.

The meeting clearly brought out a variety of new approaches. However, such important problems as the circumstances surrounding the adoption of bipedal locomotion, the possible role of dietary shifts in guiding human evolution and the nature of selection pressures favouring brain development, all remain to be resolved. □

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REVIEW ARTICLE

Calmodulin—an intracellular calcium receptor

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Calmodulin, a protein that binds calcium with high affinity and specificity, is structurally conserved and functionally preserved throughout the animal and plant kingdoms. It serves as an intracellular Ca^{2+} -receptor and mediates the Ca^{2+} regulation of cyclic nucleotide and glycogen metabolism, secretion, motility and Ca^{2+} transport. Calmodulin is also a dynamic component of the mitotic apparatus.

CALCIUM is involved in regulating a variety of cellular enzyme systems and in most types of cell motility¹⁻³. The possibility that Ca^{2+} might not act in its free ionic form but rather requires the presence of a binding protein was first suggested by Meyer *et al.*⁴ who reported that Ca^{2+} was necessary for the activity of muscle phosphorylase kinase. Bradham *et al.*⁵ demonstrated the inhibition of bovine brain adenylate cyclase by EGTA and its reversal by Ca^{2+} . Kakiuchi *et al.*⁶ and Cheung⁷ independently discovered a heat-stable protein activator of brain cyclic nucleotide phosphodiesterase and 2 years later, Wolff and Siegel⁸ purified a Ca^{2+} -binding protein from bovine brain. Weisenberg⁹ found that neurotubules were destabilised in micromolar Ca^{2+} and Bond and Clough¹⁰ found that a soluble protein fraction from haemolysates stimulated the activity of red blood cell Ca^{2+} - Mg^{2+} -ATPase. Pires *et al.*¹¹ discovered that a specific skeletal muscle myosin light chain kinase was dependent on Ca^{2+} for optimal activity.

It is now known that the same Ca^{2+} -binding protein acts as a mediator of Ca^{2+} regulation in each of these systems. Because it modulates enzyme activities in a Ca^{2+} -dependent manner, this Ca^{2+} -binding protein is now commonly known as calmodulin. As shown in Table 1, calmodulin regulates a number of fundamental cellular activities such as cyclic nucleotide and glycogen metabolism, intracellular motility (microtubules and microfilaments), and calcium transport as well as less defined Ca^{2+} -dependent protein kinases. Since we have been particularly interested in correlating the cellular and subcellular localisation of the protein with mechanistic studies carried out in defined biochemical systems, it has been necessary to determine the major physicochemical properties of the protein, to produce monospecific calmodulin antibodies, and to use these antibodies to answer a number of specific biological questions. In this review we will summarise our findings and how they integrate with the many other studies on calmodulin.

Physicochemical properties of calmodulin

Calmodulin exists as a monomer of molecular weight (MW) 17,000 and in the presence of Ca^{2+} resists denaturation (boiling, 8 M urea, 1% SDS)¹². It is well established that calmodulin contains four calcium binding sites although reported affinity and specificity of the sites vary considerably. Binding of Ca^{2+} to any one of the four equivalent sites results in a conformational change. In this state over 50% of the molecule exists in an α -helical configuration. This conformational change is required in order for calmodulin to regulate any of the enzyme systems listed in Table 1. Data from our laboratory reveal that whereas phosphodiesterase activation can occur maximally with only one Ca^{2+} bound, the effect on microtubule depolymerisation requires all four sites to be filled. Thus it is possible that the

fractional occupancy of the Ca^{2+} binding sites may dictate the systems regulated by calmodulin and help to explain how this protein can be involved in such diverse biological processes.

Calmodulin from rat¹³, cow¹⁴ and the marine coelenterate *Renilla reniformis*¹⁵ has been sequenced. It has 148 amino acid residues with no more than six conservative amino acid substitutions and only one of these in a calcium-binding domain. The protein is devoid of cysteine and tryptophan and contains a single histidine. The high ratio of phenylalanine to tyrosine (8:2) is reflected in the distinctive ultraviolet absorption profile and low molar extinction coefficient¹²; the high ratio of acidic to basic residues (2.7) accounts for the low isoelectric point (3.9)¹². The most distinctive feature of the protein molecule is that the epsilon amino group of lysine 115 is post-transcriptionally trimethylated¹⁶. This modification preserves the positive charge and may influence the interaction of calmodulin with its multiple binding proteins, by analogy with the claim that trimethylation of yeast cytochrome *c* enhances its interaction with cytochrome oxidase¹⁷.

Homology with troponin C

Many similarities exist between calmodulin and skeletal muscle troponin-C (TnC). These proteins cross-react in their respective biological systems¹⁸⁻²¹. Indeed, Kuo and Coffee²² characterised a TnC-like protein from adrenal medulla that was subsequently shown to be calmodulin. The most obvious sequence difference between calmodulin and troponin C from rabbit skeletal muscle (STnC) or bovine cardiac muscle (CTnC) is that calmodulin is 7 or 8 amino acids shorter at the amino terminal region. This region is evolutionarily variable in the troponin C family^{23,24}, suggesting limited functional importance. In addition, TnC has three extra amino acids between positions 78 and 79 of calmodulin, and a single extra residue at the C-terminus. There is 45% direct homology between the two proteins, increasing to 78% for conservative replacements (substitution by functionally similar amino acids). The region of greatest homology is in the interior portion of the molecules (amino acids 30-77) where over 70% of the residues have exact identities. Other interesting features are the fact that all eight calmodulin phenylalanines and tyrosine-99 are invariant and the remaining tyrosine (residue 138) aligns with either a tyrosine or phenylalanine. Furthermore, 8 of the 11 calmodulin glycines seem to be invariant. It can be predicted that these glycines are located at sharp turns of the α -helical chain as is true for cytochrome *c*. Calmodulin, like the troponin C's^{13,14,24} and parvalbumin²⁵, displays significant internal homology in and around the Ca^{2+} -binding sites. Sites I and III, and II and IV, contain high degrees of homology. These data would suggest that the Ca^{2+} -binding proteins evolved from a smaller ancestral precursor (which bound a single Ca^{2+}) by

gene duplication. Gene duplication is further suggested by the three residue deletion in calmodulin which occurs between binding domains II and III.

Antibody production

Calmodulin has proven to be a poor antigen, presumably because it is small, very acidic, and highly conserved in eukaryotic cells and tissues from diverse species. We have shown, however, that inoculation of animals (goat or sheep) with large quantities of homogenous calmodulin elicited a highly specific antibody^{26,27}. Andersen *et al.*²⁸ also produced calmodulin antibody using alum-precipitated protein. More recently, Wallace and Cheung²⁹ elicited antibody to dinitrophenylated calmodulin.

Our antibody, purified by calmodulin-affinity chromatography specifically inhibits the activation of cyclic nucleotide phosphodiesterase by calmodulin²⁶ and specifically precipitates ¹²⁵I-labelled or ³⁵S-labelled calmodulin from a complex mixture of labelled protein²⁷.

The availability of antibody led to the development of a radioimmuno-assay (RIA) for calmodulin²⁷. The immunoreactivity of calmodulin in all tissues and cells is considerably greater than the activity determined by phosphodiesterase activation assay. This could be because calmodulin binding proteins in cell extracts compete with phosphodiesterase for Ca²⁺-dependent binding, whereas the RIA is routinely performed in the presence of EGTA to prevent interference with calmodulin binding proteins. Alternatively there may be structural modifications (such as TML?) of calmodulin which prevent its interaction with some enzymes but allow association with others.

Regulation of calmodulin levels

The rate of synthesis and degradation of calmodulin in tissue culture or in tissue treated *in vivo* can be determined with an antibody precipitation technique²⁷. Using this we have re-evaluated claims that there is more calmodulin in virally transformed cells than in normal counterparts not expressing the viral genome^{30,31}.

In both SV-3T3 and S-NRK cells, calmodulin levels were 2–3 times higher than in non-transformed counterparts and the rate of protein synthesis, including that of calmodulin, was also increased. However, whereas the rate of total protein degradation was markedly enhanced in the transformed cells, calmodulin degradation was only slightly altered. Since the doubling time of each cell type was reflected by the rate of total protein degradation, our results suggest that the degradation of calmodulin may be constitutive. Thus the elevated calmodulin levels in transformed cells are due to a general increase in protein synthesis that accompanies the transformed state.

Further evidence of the constitutive expression of calmodulin comes from experiments in which follicle-stimulating hormone (FSH) is injected into immature or hypophysectomised rats or added to culture media containing a homogeneous population of Sertoli cells³². Although FSH stimulates RNA and protein synthesis in Sertoli cells³³, calmodulin levels are unaltered. Similarly, although the expression of every other measured protein or enzyme in the chicken oviduct has been shown to be dependent on steroid hormones, calmodulin levels per cell remain constant regardless of the hormone environment. Collectively these data suggest that regulation of calmodulin-mediated enzymes is due to alterations in the net flux or distribution of Ca²⁺ rather than to changes in the cellular content of calmodulin.

Location of calmodulin in interphase cells

The purified, monospecific antibody has been used to localise calmodulin in a variety of animal cells by adopting the indirect immunofluorescence procedure described by Fuller *et al.*³⁴. The cells were grown on sterile glass coverslips, fixed in formaldehyde and acetone, and then treated with the primary antibody: goat anti-calmodulin. After washing, the cells were exposed to a

second antibody, rabbit anti-goat globulin tagged with fluorescein. Specificity of the immunofluorescence was tested by performing four controls: (1) use of preimmune goat γ -globulin fraction as the primary antibody; (2) use of the nonabsorbed affinity column material as the primary antibody; (3) pre-absorbance of the purified anti-calmodulin with a fivefold excess of homogeneous testis calmodulin; and (4) incubation of the second antibody without the primary (anti-calmodulin) antibody. The background fluorescence of the controls was quite low with only traces of nonspecific fluorescence. In interphase PtK₂ cells calmodulin was present throughout the cytoplasm yet appeared to be excluded from the nucleus^{26,35}. Immunofluorescence also was observed in actin containing filaments extending, in some cases, across the entire length of the cell. A similar pattern of anti-calmodulin fluorescence was found in fibroblasts from humans, chick and *Xenopus* as well as rat Sertoli cells which are of epithelial origin²⁶.

The association of calmodulin with the actin containing filaments is presumably related to its role in the regulation of actomyosin ATPase activity in nonmuscle cells. Perry and associates¹¹ first described a calcium-dependent protein kinase in striated muscle that specifically phosphorylated the 20,000-MW light-chain (LC₂₀) of myosin. Subsequently, Yazawa and Yagi³⁶ purified this enzyme and discovered that it was composed of a catalytic subunit (MW ~100,000) and a calcium binding regulatory subunit (MW 20,000). This regulatory subunit was later identified by Yagi *et al.*³⁷ as calmodulin. Independently, Waisman *et al.*³⁸ described a similar skeletal muscle kinase that was regulated by calmodulin. Chick gizzard, as a form of smooth muscle, was subsequently studied with regard to the regulation of myosin ATPase activity. Again Dabrowska *et al.*³⁹ found that the myosin light chain kinase consisted of a catalytic and regulatory component, the latter being calmodulin. Even the myosin light chain kinase present in nonmuscle cells has been shown to be a calmodulin-dependent enzyme. This has been most carefully examined in blood platelets^{39,40} and baby hamster kidney cells¹⁸ in tissue culture. Taken together, these observations argue that calcium binds calmodulin and this complex activates the kinase to phosphorylate myosin (LC₂₀). This results in a conformational change to allow actin to come into contact with the myosin head group thus stimulating myosin ATPase activity. It has been demonstrated that such interaction results in increased tension development and presumably motility.

Localisation of calmodulin during mitosis

In mitotic cells, anti-calmodulin fluorescence was associated with the mitotic apparatus of 3T3 and PtK₂ cells²⁶ in all phases of mitosis (Fig. 1). Fluorescence was most intense at the spindle pole and projected towards the chromosome in a distribution

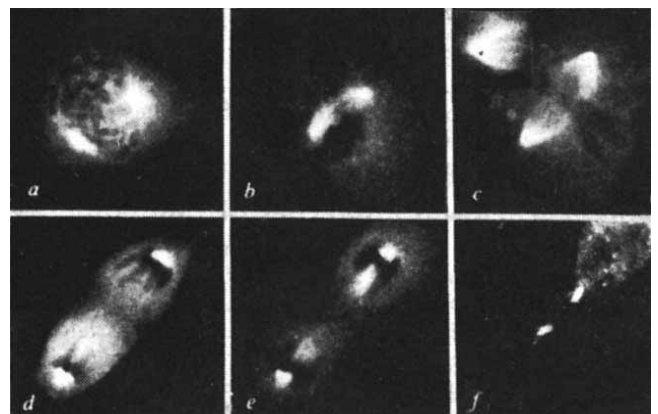


Fig. 1 Distribution of calmodulin during mitosis visualised by indirect immunofluorescence microscopy. Monolayers of rat kangaroo PtK₂ cells were plated on glass coverslips and prepared for immunofluorescent localisation of calmodulin as described by Welsh *et al.*⁴¹. Calmodulin antibody was raised in sheep and purified by affinity chromatography on calmodulin-Sepharose²⁶. The fluorescein-labelled second antibody was rabbit anti-sheep. *a*, Prophase; *b*, prometaphase; *c*, metaphase; *d*, anaphase; *e*, late anaphase; *f*, telophase.

that gave the appearance of fibres. However, the pattern of calmodulin-specific fluorescence did not traverse the metaphase plate but instead terminated on or before reaching the chromosomes. Only in very late anaphase was calmodulin-specific fluorescence demonstrated transiently in the intrazonal region but again extremely close to the chromosomes. These data were independently confirmed by Andersen *et al.*²⁸ using an anti-calmodulin prepared in rabbits.

The distribution of calmodulin and tubulin was similar but not identical during mitosis⁴¹. Treatment of cells with colcemid disrupted the mitotic apparatus and caused a disappearance of specific fluorescence both for tubulin and for calmodulin. On the other hand, cytochalasin B, which under some circumstances dissociates microfilaments, had no effect on the mitotic apparatus. Similarly, no effect was noted on specific fluorescence patterns of tubulin or calmodulin. Nitrous oxide treatment causes the disorganisation of the mitotic spindle but does not result in disassembly of spindle microtubules. Treatment of Chinese hamster ovary cells with nitrous oxide disrupted spindle structure as seen with either tubulin or calmodulin specific fluorescence.

Finally, exposure of cells to low temperatures has been shown to effectively disrupt certain populations of microtubules in tissue culture. Intrazonal microtubules depolymerise when mitotic cells are incubated at 4 °C whereas the kinetochore-to-pole microtubule bundles are unaffected⁴². Experiments performed with anti-tubulin fluorescence confirmed previous studies in that the pole-to-pole microtubules were disrupted leaving only the cold stable pole-to-chromosome microtubules. On the other hand, such treatment did not affect the localisation of calmodulin. These results suggest that calmodulin might be preferentially associated with the chromosome-to-pole microtubules and thus, might be involved in the assembly/disassembly of microtubules that occurs during the movement of chromosomes from the metaphase plate to the spindle poles.

Brinkley and Cartwright⁴³ utilised electron microscopy to quantitate the number of microtubules present in serial sections of mitotic cells. When these data were compared to the localisation data for calmodulin, it was found that in all instances and in all phases of mitosis, the high concentrations of calmodulin were associated with low or nonexistent numbers of microtubules. Not only did this hold true in metaphase and early anaphase, but at very late anaphase, corresponding to the transient association with calmodulin on either side of the separating chromosomes, no microtubules were found in this region but were restricted to the portion of the cell corresponding to the developing cleavage furrow.

A role for calmodulin in assembly/disassembly of microtubules

It has long been known that microtubules are sensitive to calcium, although the concentration of calcium required for depolymerisation of microtubules has been somewhat controversial^{9,44}. To determine whether calmodulin had any effect on microtubule polymerisation and might represent a protein involved in the assembly/disassembly processes, microtubule protein was isolated from rat brain by a modification of the temperature-dependent assembly/disassembly method described by Borisy *et al.*⁴⁵. In order to control calcium concentrations, the calcium/EGTA buffer system described by Perrin and Sayce⁴⁶ and modified by Potter and Gergely⁴⁷ was used. Calmodulin alone (at 0.4 μ M free calcium) or 11 μ M of calcium alone caused only a slight reduction in the rate or degree of microtubule protein polymerisation⁴⁸. However, the addition of 11 μ M calcium combined with calmodulin abolished microtubule assembly. Thus, the calcium-calmodulin complex prevented the *in vitro* assembly of microtubule protein. In a complementary series of experiments, the calcium-calmodulin complex promoted complete disassembly of microtubules. Similar data have been reported by Kumagai and Nishida⁴⁹ using slightly different conditions and monitoring microtubule assembly/disassembly by viscometry.

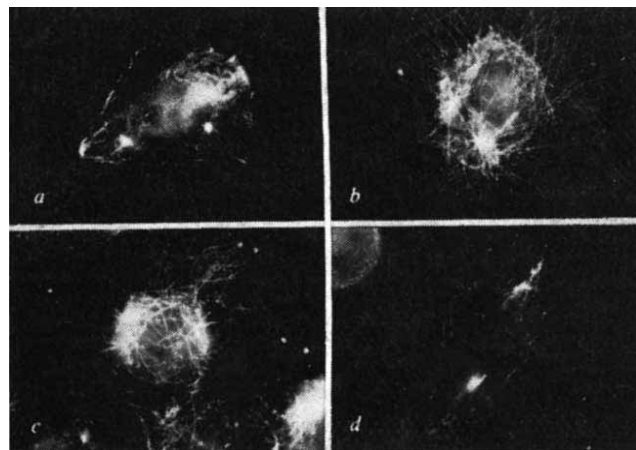


Fig. 2 Effect of Ca^{2+} -calmodulin on microtubule assembly in lysed interphase 3T3 cells. Swiss mouse 3T3 cells were treated with colcemid to disrupt the cytoplasmic microtubule network and lysed with 0.05% Triton X-100. Phosphocellulose purified 6S tubulin was added to the lysed cells and allowed to incubate for 15 min at 37 °C. Microtubules were visualised by indirect immunofluorescence microscopy using rabbit anti-tubulin³⁴. This system was developed by B. R. Brinkley *et al.*⁴⁵. *a*, Colcemid-treated cells. A few short microtubules can be seen at the cell periphery. Note the highly fluorescent spots that correspond to the centrosomal regions of the cell. *b*, Colcemid-treated cells following addition of 6S tubulin. Numerous long microtubules can be seen which have nucleated from the organising centres seen in (*a*). *c*, Colcemid-treated cells following incubation with 6S tubulin and 11 μ M free Ca^{2+} . Again numerous long microtubules are evident. *d*, Colcemid-treated cells incubated with 6S tubulin, 11 μ M Ca^{2+} and calmodulin. Microtubule assembly from the centrosomes has been prevented.

Collectively these immunofluorescence, biochemical and ultrastructural observations have led us to propose that calmodulin functions in mitosis to depolymerise the microtubules in the polar region, shortening the kinetochore-to-pole microtubules and allowing movement of chromosomes to the poles. However, it was unclear from our studies whether calmodulin acted directly on 6S tubulin, capped the assembly end of microtubules or interacted with the microtubule associated proteins. Therefore, we have established another system to investigate the potential role of calmodulin in polymerisation of microtubules.

In the interphase cell, microtubules arise from a single organising centre associated with the centrosome⁵⁰. It seems likely, therefore, that if the cytoplasmic microtubule network could be dissociated, and the depolymerised tubulin removed from the cell, this organising centre could be used to nucleate assembly of microtubules. If cells were incubated in the absence of tubulin a fluorescent organising centre could be seen, but no microtubules could be found emanating from the centre (Fig. 2). Addition of 6S tubulin resulted in a large number of microtubules arising from each organising centre and lengths reached up to 35 μ m. If calmodulin, in the absence of calcium alone or 11 μ M was added, microtubule assembly proceeded without change (Fig. 2). However, if calcium and calmodulin were added together, microtubule assembly was completely prevented. Thus, this additional procedure also demonstrated a role for calmodulin in microtubule assembly/disassembly. Microtubules examined by electron microscopy were shown not to contain high molecular weight microtubule-associated proteins. This meant that if microtubule-associated proteins were important for assembly, they would have to have been present in the lysed cell system.

Immuno-electron microscopy using horseradish peroxidase-coupled Fab fragments of anti-calmodulin showed a specific reaction product in close proximity to the centrioles and to the kinetochores (Fig. 3). In addition, calmodulin was localised in smooth endoplasmic reticulum that was parallel to the microtubule bundles in the half spindle, and also in association with membrane vesicles and mitochondrial membranes. No calmodulin was found in direct association with the pole-to-pole

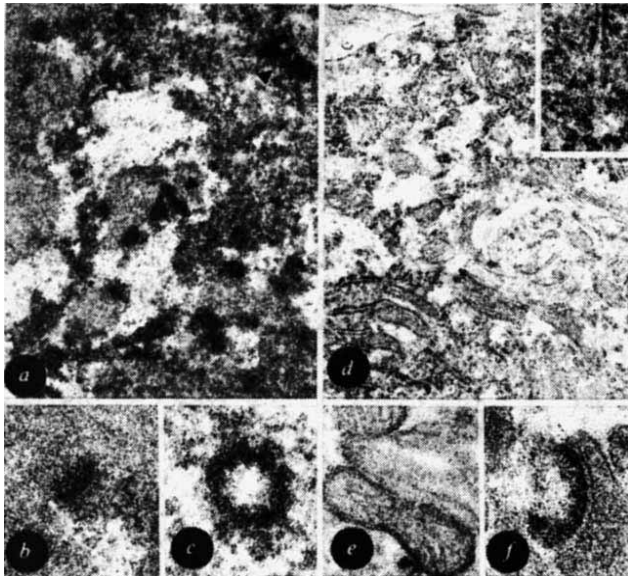


Fig. 3 Immunoelectron microscopic localisation of calmodulin. Calmodulin was localised in mitotic 3T3 cells and in rat cerebellar Purkinje cells using a calmodulin-specific Fab fragment coupled to horseradish peroxidase. The procedure was identical to that described by Lin *et al.*⁸⁶ and the experiments were performed in collaboration with Thomas Lin and B. R. Brinkley. *a*, A mitotic cell showing calmodulin reaction product around centrioles (triangle in upper left), kinetochores (triangle in middle), and some spindle microtubule bundles ($\times 11,500$). *b*, A cross-section of a centriole from another mitotic cell showing heavy deposition of reaction product around the microtubule areas. The microtubules are not evident since the sections were not counterstained. The centre of the centriole is unstained ($\times 44,400$). *d*, One Purkinje cell body from rat cerebellum showing calmodulin reaction product on free and bound ribosomes, plasma membrane, smooth endoplasmic reticulum and mitochondrial membranes ($\times 11,000$). *e*, High magnification from another area of the same cell. Dense reaction product is seen on membrane bound ribosomes and free polysomes ($\times 25,000$). *f*, High magnification of mitochondria from a neuronal dendrite. Calmodulin reaction product can be seen to decorate both outer and inner mitochondrial membranes ($\times 30,000$). *f*, A synapse from the cerebellar cortex showing heavy deposition of reaction product on a postsynaptic membrane ($\times 46,000$).

microtubules. These data support the information gained from the lysed cell system and suggest at least three possibilities for the role of calmodulin in the mitotic apparatus. First, calmodulin might directly regulate microtubule assembly/disassembly in the centrosomal regions. Second, calmodulin may regulate actomyosin. Herman and Pollard⁵¹ have clearly demonstrated by immunofluorescence microscopy that the pattern of actin in the mitotic apparatus is very similar to what we have found for calmodulin. Unfortunately, the presence of myosin in the mitotic spindle is somewhat less clear. Nevertheless, because of the role of calmodulin in the regulation of actomyosin ATPase activity in interphase cells^{18,26,35,37,39,40,52}, this must be considered and is clearly a testable hypothesis. The third possibility is that calmodulin may be regulating membraneous calcium pumps. In almost all instances calmodulin was associated with various types of membranes in the mitotic cell. Since this protein, in association with calcium, has been shown to regulate the red cell calcium pump⁵³, the sarcoplasmic reticulum calcium pump⁵⁴ and the endoplasmic reticulum calcium pump, it is possible that one of the major roles of calmodulin in the mitotic apparatus is to regulate the concentration of calcium in the microenvironment of the spindle.

Additional roles for calmodulin

Multiple roles for calmodulin are suggested by its multiple localisation in all tissues, best illustrated in the rat cerebellum (Fig. 3). Calmodulin was concentrated in Purkinje and glial cell bodies where it was predominantly associated with free and bound ribosomes, the nuclear envelope, coated membranes of smooth and rough endoplasmic reticulum, mitochondrial and

plasma membranes⁵⁵. Calmodulin was also found in the neuronal dendrites of the Purkinje cells, in which it coated the surface of smooth endoplasmic reticulum, small vesicles, and inner and outer mitochondrial membranes. In addition, granular calmodulin could be found in the central portion of the neuronal process. However, the neurotubules showed no specific association with calmodulin. The localisation in the neuronal process is in keeping with the suggestion by Iqbal and Ochs⁵⁶ that calmodulin may be important in mediating fast axonal transport. Calmodulin was also found by us, by Grab *et al.*⁵⁷ and by Wood *et al.*⁵⁸ on the inner surface of the postsynaptic membrane present in a variety of nervous system structures. Together these observations suggest that calmodulin is synthesised on both free and bound ribosomes and discharged into the cytosol. A large portion of this protein becomes associated with intracellular membranes. In addition, some calmodulin seems to be transported along the neuronal process to the postsynaptic membrane. At the postsynaptic membrane calmodulin may mediate the calcium effects on synaptic transmission and thus have a role in the release of neurotransmitters⁵⁹.

Satir and colleagues, in collaboration with our laboratory have revealed the presence of calmodulin in the basal bodies of cilia from *Paramecium*, *Tetrahymena* and the freshwater mussel *Elliptio*, and Jamieson *et al.* have shown that calmodulin is a constituent of the axonemal complex of cilia⁶⁰. These data suggest that the effect of calcium on ciliary arrest might involve calmodulin. This possibility is supported by the localisation of calmodulin in mammalian sperm to the acrosome, in the mid-piece associated with the fertilisation ridge and on both ends of the axonemal complex in the tail⁶¹. Since it seems that assembly of the axoneme can be controlled at both ends, calmodulin may have a role similar to that which it has in the mitotic apparatus during karyokinesis. In addition, the dynein of mammalian spermatozoa is similar to myosin in that it contains a Ca^{2+} -dependent ATPase⁶². It is, therefore, possible that calmodulin may modulate the dynein ATPase activity. In brief, calmodulin may be involved in promoting the attenuation of motility.

It has also been found to be associated with coated vesicles involved in receptor-mediated endocytosis. In collaboration with Linden and Roth⁶³, we have discovered that Ca^{2+} -calmodulin specifically binds to isolated coated vesicles with a dissociation constant of 10^{-8} M. The binding is calcium-dependent. In fact, half-maximal binding occurs at $2.4 \mu\text{M}$ free Ca^{2+} which is identical to the K_d of calmodulin for Ca^{2+} (ref. 12). The predominant calmodulin binding protein is clathrin. The biological function of calmodulin present in coated vesicles is unknown, but it is reasonable to suppose that it modulates the vesicle's calcium-dependent ATPase.

Table 1 Calmodulin-mediated processes

Process	Selected refs
<i>Cyclic nucleotide metabolism</i>	
Phosphodiesterase	6, 7, 71
Adenyl cyclase	71
<i>Protein phosphorylation</i>	
Membrane proteins	72, 73
Cytoplasmic proteins	74
<i>Myosin light chain kinase</i>	
Skeletal muscle	37
Smooth muscle	52
Non-muscle	18, 39, 40
Stress fibre localisation	22, 23
<i>Microtubule assembly/disassembly</i>	35, 41, 48
<i>Glycogen metabolism</i>	
Phosphorylase kinase	67, 75, 28, 29, 76, 77
<i>Calcium flux</i>	
Ca^{2+} - Mg^{2+} ATPase	78, 79, 80
Ca^{2+} transport	53, 54, 56
<i>Secretion</i>	
Intestinal ion secretion	81
Neurotransmitter release	57
<i>Other enzyme systems</i>	
NAD^{+} kinase	82
Tryptophan 5'-monooxygenase	83
Phospholipase A_2	84

Receptor-mediated calcium regulation

The mechanism of action of Ca^{2+} is different from that of other metal ions. Indeed, the role of Ca^{2+} seems to be more similar to the regulatory actions of cyclic nucleotides which produce multiple metabolic effects by binding to the regulatory subunits of cyclic AMP-dependent protein kinase and thereby activating the enzyme. The specificity of the cyclic nucleotide system of any cell is determined at two levels. The first is by the receptors (for hormones and so on, that elevate the intracellular content of cyclic AMP) the membrane is endowed with. The second is which substrate(s) for the protein kinase that the cell possesses. By analogy to cyclic AMP, it has been proposed that Ca^{2+} is an intracellular second messenger¹. Continuing this analogy, calmodulin may be considered an intracellular receptor for Ca^{2+} . Evidence consistent with this is that (1) in non-muscle cells it represents the major high affinity Ca^{2+} -binding protein; the affinity for Ca^{2+} is approximately equal to the estimated intracellular free- Ca^{2+} concentration, suggesting that physiologically active cellular Ca^{2+} is calmodulin bound; (2) no alternate function (that is, enzymatic or structural) has been demonstrated; (3) it has been structurally conserved and functionally preserved throughout both animal and plant kingdoms, suggesting that it is involved in numerous cellular activities which require an invariant mediator; (4) it would be predicted that, in order to account for its many cellular activities, calmodulin would have multiple 'acceptor' proteins and a number of the major forms have been identified (Table 1). Many more probably exist. Studies by Grand and Perry have shown that each mammalian tissue possesses a distinct set of these proteins⁶⁴, through which the specificity of Ca^{2+} action is

presumably determined. Amplification of a Ca^{2+} signal, like a cyclic AMP signal, can be accomplished through the activation of specific protein kinases which in turn may affect a number of additional enzyme activities. It is likely that the discovery of calmodulin and the developing understanding of how this protein is involved in the regulation of numerous calcium mediated events will provide a link in our understanding of the interaction of calcium and cyclic nucleotides in the control of cellular metabolism.

At the moment most of the data concerning calmodulin regulation must be considered as phenomenological. What is now required is a concerted effort to understand the chemical basis for each of calmodulin's actions. This will require isolation and purification of the enzyme systems regulated by this protein. Indeed, some success has already been achieved along these lines. Phosphodiesterase^{65,66}, myosin light chain kinase^{37,52}, phosphorylase kinase⁶⁷ and ATPase^{68,69} have all been purified and calmodulin stimulated adenylyl cyclase⁷⁰ is reported to have been partially purified. Even when one understands the interaction of calmodulin with these enzyme systems, it will be necessary to discover how hormones regulate the activity of calmodulin. Since this protein seems to be constitutively synthesised it is likely that the activity is regulated by the concentration of available calcium.

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ARTICLES

Mutations which alter the function of the signal sequence of the maltose binding protein of *Escherichia coli*

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The maltose binding protein of Escherichia coli is secreted into the external periplasmic compartment of the cell by virtue of an amino-terminal signal sequence. Using DNA sequencing, we have determined the precise nature of mutations in the signal sequence which prevent the export of the maltose binding protein, causing it to accumulate in the cytoplasm in its precursor form. In most cases, the change of a single hydrophobic or uncharged amino acid to a charged amino acid within the signal sequence is sufficient to block the secretion process.

MOST secreted proteins in both prokaryotic and eukaryotic cells are initially synthesised with an amino-terminal extension of some 15 to 30 amino acids which is subsequently excised. It has been proposed that this 'signal sequence' is responsible for initiating passage of the polypeptide through the membrane while it is still a nascent chain attached to the ribosome¹⁻³. The exact mechanism by which this is accomplished, and the nature of the putative receptor in the membrane, are unknown. Among the different signal sequences thus far determined, there seems to be no significant primary sequence homology⁴. However, one feature common to all signal sequences is a central core of 9-24 predominantly hydrophobic amino acids. In most cases, this is preceded by one or more charged residues, usually lysine and/or arginine. One approach to defining those features of the signal sequence which are essential for initiating protein export is to generate mutations which alter the signal sequence of a secreted protein, resulting in the failure of the cell to export the protein from the cytoplasm.

The Gram-negative bacterium *Escherichia coli* provides a convenient system for isolating mutants which affect secretion, as genetic manipulations of it are relatively easy and many of its proteins are localised in the cell envelope, and, therefore, must be secreted through the cytoplasmic membrane. Evidence exists that signal sequences are involved in the export of proteins to at least two of the bacterial envelope compartments⁵. These compartments are the outer membrane and an aqueous layer located between the two membranes, called the periplasmic space.

One of the proteins found in the periplasmic space of *E. coli* is the maltose binding protein (MBP), a key component of the maltose transport system⁶. We recently described a selection procedure which enabled us to isolate mutants of *E. coli* which fail to secrete the MBP into the periplasm⁷. In the mutants we obtained, the MBP accumulates in the cytoplasm in its higher molecular weight precursor form. From the genetic data, we suggested that the mutations in these strains resulted in single amino acid substitutions in the MBP signal sequence. We now report the DNA sequence coding for the first 50 amino acids of

the wild-type MBP precursor, the identification of the signal sequence which was done through partial amino acid sequence determination, and the DNA sequence changes for several MBP export-defective mutants.

Partial determination of the MBP signal sequence using a *malE-lacZ* hybrid protein

Because the MBP precursor does not accumulate to appreciable amounts in wild-type cells, material for determining the MBP signal sequence was obtained from another source. We had previously isolated *E. coli* strains in which the *malE* gene encoding the MBP is genetically fused to the *lacZ* gene encoding the enzyme β -galactosidase⁸. The resultant hybrid gene codes for a hybrid protein which has at its amino-terminus an amino-terminal portion of the *malE* gene product and, at its carboxy-terminus, enzymatically active β -galactosidase. One such strain, PB4-81, synthesises a hybrid protein which is cytoplasmic and which seems, from the genetic and biochemical data, to include only a very small, amino-terminal portion of the *malE* gene product. Consequently, we suspected that this protein contained only a portion of the MBP signal sequence. Thus, this protein should provide material for a partial signal sequence determination.

The determination of the PB4-81 hybrid protein sequence was made possible by techniques developed for the sequence analysis of a hybrid protein between β -galactosidase and the *lac* repressor⁹. A partial signal sequence for alkaline phosphatase had been determined previously in this way¹⁰. The procedure used to generate hybrid proteins must result in the removal of at least the first 17 amino acids of wild-type β -galactosidase, thus eliminating the first methionine residue. If the amino acid sequence which replaces the normal β -galactosidase amino-terminus does not contain any methionine (other than the initiating methionine), only one new cyanogen bromide fragment (CNBr2*) should result from treatment of the hybrid protein. This proved to be the case with the hybrid β -galactosidase from strain PB4-81. Sequence analysis of the CNBr2* indicated that the first 14 residues are not derived from β -galactosidase (Table 1). These residues are derived from the

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Table 1 Sequencer analysis of the *malE-lacZ* hybrid protein from strain PB4-81

Amino acid identified				Amino acid identified			
Residue no.*	Intact protein†	CNBr2*‡	CNBr2* T4§	Residue no.*	Intact protein†	CNBr2*‡	CNBr2* T4§
1 Met	+			*16 Pro	+	+	
2 Lys	+	+		*17 Gly	+	+	
3 Ile	+	+		*18 Val	+	+	
4 Lys	+	+		*19 Thr	+	+	
5 Thr		+		*20 Gln		+	
6 Gly	+	+		*21 Leu	+	+	
7 Ala	+	+		*22 Asn		+	
8 Arg		+		*23 Arg			
9 Ile	+	+	+	*24 Leu		+	
10 Leu	+	+	+	*25 Ala		+	
11 Ala	+	+	+	*26 Ala		+	
12 Leu	+	+	+	*27 His		+	
13 Ser		+		*28 Pro			
14 Ala	+	+	+	*29 Pro		+	
15 Ser		+		*30 Phe		+	
				31 Ala		+	

All sequencing was done using 0.1 M Quadrol in a Beckman 890C sequencer. The peptides were sequenced using a dual wash program. The intact carboxymethylated protein was sequenced with a program similar to that of Hunkapiller and Hood. All other procedures were as described previously, except that recently identification was also carried out with HPLC. + Indicates that the amino acid indicated was detected. A blank indicates either an ambiguous result or that it was not possible to detect the amino acid by this technique.

* Amino acids identified with an asterisk correspond to residues 19–34 of native *E. coli* β -galactosidase.

† The hybrid β -galactosidase was purified by the same procedure as for the *lac* repressor- β -galactosidase hybrid. Strain PB4-81 was grown in a 150-l New Brunswick fermentor at 30°C in medium 63 containing 1% glycerol and 0.2% maltose. The hybrid protein was purified 40-fold with a yield of 36% based on β -galactosidase activity. It was greater than 90% pure on SDS gels²² and migrated with a mobility close to that of wild-type β -galactosidase.

‡ The hybrid protein was carboxymethylated and digested with cyanogen bromide as described elsewhere²¹. The CNBr2* peptide was purified using CM-cellulose and SP-Sephadex ion-exchange chromatography in 8 M urea and gel filtration on Sephadex G-50 in 30% acetic acid²¹. The radioimmunoassay used to monitor purification on CNBr2* has been described previously⁹. The yield of CNBr2* from purified hybrid protein was 25%. The elution volume on Sephadex G-50 suggested a size of about 90 residues. Amino acid composition data indicated that the PB4-81 CNBr2* contains lysine whereas wild-type CNBr2 has no lysine.

§ To confirm the presence of serine at residue 14 of CNBr2*, the peptide was cleaved with trypsin and the resulting peptides purified. Amino acid analysis of CNBr2* (corresponding to residues 9–23 of the intact protein) indicated two residues of serine and three residues of leucine. Details of the protein and peptide purifications and sequence analysis will be presented elsewhere (A.V.F., I.Z., P.J.B. Jr and J.B. in preparation).

amino-terminus of the protein to which β -galactosidase has been fused, in this case, the *malE* gene product. Two factors suggested that these 14 residues, along with the amino-terminal methionine from the hybrid protein, corresponded to the first 15 amino acids of the MBP precursor. First, this 15-residue sequence, with three basic residues among the first eight followed by several predominantly hydrophobic amino acids, is similar to other signal sequences. Second, none of this sequence was encountered when the protein sequence of the amino-terminal 25 amino acids of the mature MBP was determined (data not shown, see Fig. 1 legend).

DNA sequence of the wild-type *malE* signal sequence region

Cleavage of plasmid pHCS, which carries the *malB* operon (see Fig. 2), with the restriction endonuclease *EcoRI*, generates a fragment of 1,227 base pairs, comprised of 850 base pairs from the *malB* region of the *E. coli* chromosome and 377 base pairs derived from the vector (plasmid pBR322) (Fig. 2). This *EcoRI-EcoRI* hybrid fragment includes the promoter-proximal portion of the *malE* gene which codes for the MBP signal sequence region. Beginning with this fragment, a region of 600 nucleotides extending from the *EcoRI* cleavage site in the *malK* (ref. 11) gene in the direction of the *malE* gene has been sequenced on both strands, using the dideoxy/nick-translation technique of Maat and Smith¹². This region includes the entire regulatory interval between the two *malB* operons and will be described elsewhere (H.B., in preparation).

We have taken the *EcoRI* cleavage site in *malK* on the strand encoding the MBP as the origin for numbering purposes. Starting from this point and examining the sequence towards the *malE* gene, the first ATG initiation triplet which is not soon followed in phase by a nonsense triplet is at nucleotides 457–459. Just before this point, at nucleotides 443–447, there is a 5-nucleotide sequence complementary to the 3'-OH end of the 16S rRNA (Shine and Dalgarno sequence¹³). This sequence suggests the existence of a translation start which is used *in vivo*. In fact, nucleotides 457–498 correspond to the first 14 amino acids of the MBP precursor, as determined from the protein sequence of the PB4-81 *malE-lacZ* hybrid protein described

above. Nucleotides 535–600 correspond to the first 22 amino acids of the mature MBP. Consequently, we deduce that the complete MBP signal sequence, that part of the MBP precursor which is excised to yield the mature protein, must be 26 amino acids long and encoded by nucleotides 457–534 (summarised in Fig. 1 legend).

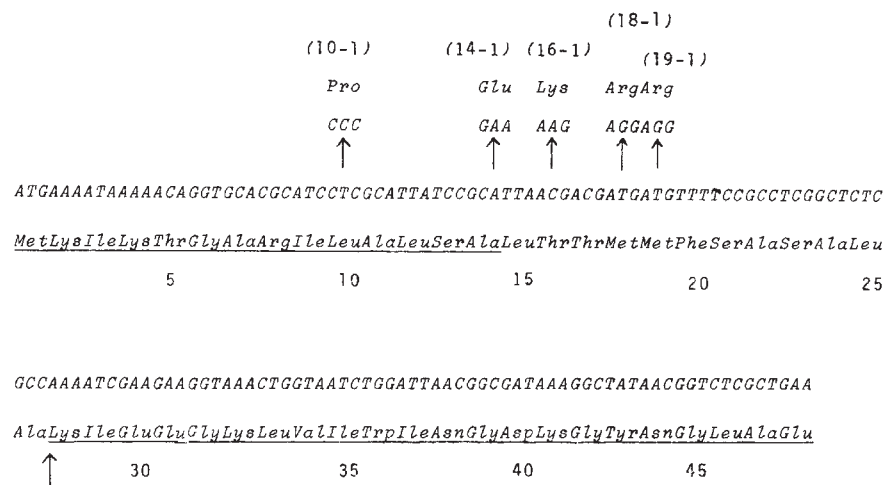
Note that the serine at residue 15 of the PB4-81 hybrid protein (Table 1) is not derived from either the *malE* or the *lacZ* gene; neither can it arise from a simple fusion of the *malE* and *lacZ* genes within a codon. This serine residue may have been generated during the *in vivo* gene fusion technique which used bacteriophage Mu to obtain the *malE-lacZ* hybrid gene⁸.

Sequencing the DNA of the *malB* region of MBP export-defective mutants

From the DNA sequence of the regulatory interval between the two *malB* operons, we identified a cleavage site for endonuclease *HinfI* at nucleotide 406, and a cleavage site for *AvaII* at nucleotide 445. Restriction analysis revealed that the enzymes *HinfI* and *AvaII* cleaved the *EcoRI-EcoRI* hybrid fragment of plasmid pHCS at only one site each, this being the one identified from the sequence (Fig. 2). Simultaneous digestion of the hybrid fragment with *HinfI* and *AvaII* yields two large fragments, as well as one small 39-base pair fragment corresponding to nucleotides 407–445 on the numbered strand.

To determine the sequence of the putative *malE* signal sequence mutations we had previously isolated, we used the technique developed by Sanger and co-workers¹⁴ with the 39-base pair *HinfI-AvaII* fragment serving as a primer. The advantages of this technique were as follows. (1) The genetic data on these mutations indicated that they were located early in the *malE* gene⁷. (2) These mutations were isolated directly on a transducing phage carrying the region of interest. Thus, phage DNA for each of the mutants could be used as template for the sequencing procedure after strand separation. (3) The *AvaII* end of the *HinfI-AvaII* fragment is located 11 nucleotides from the beginning of the *malE* gene. This allowed us to read approximately 150 nucleotides into the *malE* gene in a single experiment.

Using this procedure, we have verified the wild-type DNA



sequence for the first 144 nucleotides of the *malE* gene which had previously been obtained by the dideoxy/nick-translation technique (Figs 1, 3).

Phenotype of MBP export-defective mutants

The mutations thought to result in MBP signal sequence alterations were initially obtained in a *malE-lacZ* hybrid gene carried on the λ transducing phage, λ p72-47 (ref. 7). In this case, the hybrid gene encodes a hybrid protein that includes a substantial portion of the MBP, including the entire MBP signal sequence⁸. The mutations, which were located in the *malE* portion of the hybrid gene, were subsequently recombined into a wild-type *malE* gene on the bacterial chromosome. The resulting recombinants are *Mal*⁻ when scored on maltose tetrazolium agar, indicating that their ability to utilise maltose as a carbon source has been impaired. When they are induced for the expression of the *malEFG* operon, they accumulate the MBP precursor in the cytoplasm. However, these strains are not absolutely defective, for they grow slowly on maltose minimum medium and export a small amount of native MBP to the periplasm (see ref. 7). Furthermore, the mutants vary in their ability to utilise maltose, indicating that they may each affect the secretion of MBP to different extents (Table 2).

DNA sequence alterations in MBP export-defective mutants

Using the same sequencing procedure described above, we determined the DNA sequence of the promoter-proximal 144 nucleotides of the *malE* gene for nine spontaneous, independently isolated MBP export-defective mutants. The DNA used as template was obtained from the λ transducing phage on which the mutations were initially isolated (derivatives of λ p72-47).

Each of the nine mutants sequenced exhibited an alteration in the region of the *malE* gene corresponding to the MBP signal sequence. Five different alterations were recognised (Fig. 7). The transition CTC (Leu) to CCC (Pro), resulting in a change in residue 10 of the MBP precursor, occurred in one mutant. The transversions GCA (Ala) to GAA (Glu) at position 14, ACG (Thr) to AAG (Lys) at position 16, and ATG (Met) to AAG (Arg) at positions 18 and 19 were encountered 3, 1, 2 and 2 times, respectively.

Discussion

We have presented here the DNA sequence of the MBP signal sequence and of mutant signal sequences which result in defective export of the maltose binding protein. The MBP signal sequence is similar to signal sequences which have been determined for other exported proteins in both prokaryotic and eukaryotic systems. Of the 26 amino acids comprising the MBP signal sequence, almost two-thirds are hydrophobic residues. Furthermore, all three charged residues present (two lysines and an arginine) are clustered in the first eight residues. Following

the arginine at residue 8, only the neutral amino acids serine and threonine interrupt the hydrophobic nature of the MBP signal. The amino acid just before the cleavage site in the MBP precursor is alanine. This is consistent with other signal sequences, where the residue here usually has a side chain with no more than one carbon moiety⁴.

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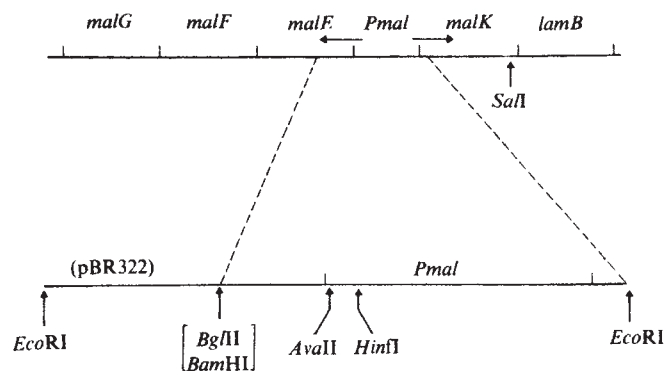
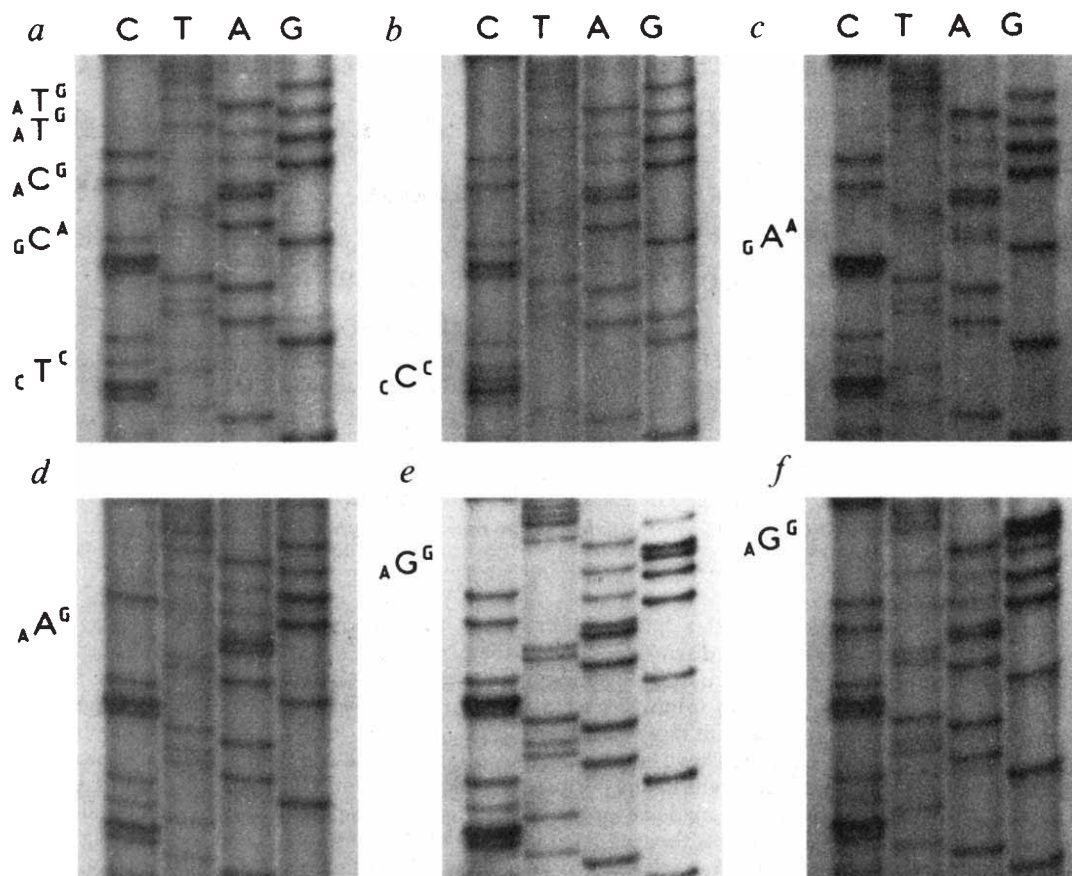


Fig. 2 The *malB* region of *E. coli* includes the *malE* gene and four other genes whose products are also components of the maltose transport system²³. These are organised into two operons orientated in opposite directions and which diverge from the promoter region designated here as *Pmal* (ref. 24). The 1,227-base pair *EcoRI-EcoRI* hybrid fragment shown above is obtained by *EcoRI* digestion of plasmid pHC6. This plasmid carries a bacterial fragment of 1,750 base pairs which extends between cleavage sites in the *malB* region for restriction endonucleases *SalI* (in *malK*) and *BglII* (in *malE*)²⁵. This fragment was inserted between cleavage sites for *SalI* and *BamHI* in pB322²⁶. Details on the construction and characterisation of plasmid pHC6 will be presented elsewhere (J. M. Clement, D. Perrin and J. Hedgpeth, in preparation). Sites for cleavage of the hybrid fragment by *AvaII* and *HinfI* are also indicated (see text).

The isolation of mutants with well defined alterations in the signal sequence of a secreted protein and in an outer membrane protein (see accompanying paper¹⁵) which result in the failure of the cell to secrete these proteins, provides the first genetic proof for the role of the signal sequence in the initial steps of protein secretion. Other workers on bacterial systems have suggested that the basic residues early in the signal sequence are responsible for the initial interaction of the precursor with the negatively charged inner surface of the cytoplasmic membrane^{16,17}. Subsequently, according to this model, the hydrophobic portion of the signal begins to penetrate the membrane as the first step in the translocation process. Four of the five mutations we have characterised introduce a single charged residue into the hydrophobic core of the MBP signal sequence. Three of these mutations were found at least twice in independently isolated mutants. This strongly supports the notion that the hydrophobicity of the signal sequence has a key role in initiating protein export through the membrane. In the remaining case,

Fig. 3 Composite figure comparing autoradiograms of sequencing gels for the wild-type *malE* gene and the *malE* signal sequence mutations. Only the relevant parts of the gels are shown. *a*, The wild-type sequence. Points along the sequence where mutations were encountered are indicated on the left. The enlarged letter indicates the exact nucleotide that is altered in the mutants. *b-f* Represent the five classes of signal sequence mutations which we obtained in this analysis. The mutations shown in *e* and *f* correspond to those present in *E. coli* strains PB1101 and PB1102, respectively, as described in ref. 7.



the substitution of proline for leucine at residue 10 represents an exchange of hydrophobic residues. Proline does have the property of interrupting α -helices in polypeptide chains¹⁸, so it is conceivable that secondary structure of the signal sequence plays a part in the initial steps of secretion.

The changes observed have differing effects on MBP secretion (Table 2). The two mutations with the strongest effects are 18-1 and 19-1, which change a hydrophobic to a charged amino acid. The three remaining mutations, which do not cause such a dramatic reduction in growth on maltose, involve a hydrophobic to hydrophobic amino acid change (10-1) and an uncharged to a charged amino acid change (14-1 and 16-1). These results are also consistent with the notion that the overall hydrophobic nature of this region is the most important feature for the secretion process.

All of the mutations we have obtained in the MBP signal sequence are point mutations which readily revert⁷. We hope to obtain additional information on the important structural features of the MBP signal sequence through a sequence analysis of such revertants.

Table 2 Growth properties of *malE* signal sequence mutants

Mutant no.	Growth on maltose minimal medium
Wild-type	++++
10-1	++
14-1	++
16-1	+++
18-1	±
19-1	+

The mutants examined here are given numbers according to the amino acid position in the protein. Mutants 18-1 and 19-1 correspond to mutants 1101 and 1102, respectively, as described in ref. 7. The growth phenotype was determined by observing colony size on maltose minimal agar at 37°C after 1 day. The hierarchy of growth rates corresponded exactly to the gradient of colours seen on maltose tetrazolium agar. For example, the wild-type parent forms white colonies, whereas mutant 18-1 formed the darkest red colonies on this agar. Mutant 16-1 forms a pink colony on the same agar, but apparently secretes enough MBP to the periplasm to allow near normal growth on maltose minimal media.

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Sequence analysis of mutations that prevent export of λ receptor, an *Escherichia coli* outer membrane protein

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The amino-terminal signal sequence is required for initiation of transmembrane protein transfer of the Escherichia coli λ receptor protein. Mutations leading to insertion of charged amino acids into or deletion of amino acids from the hydrophobic segment of this sequence prevent export of this outer membrane protein.

TRANSMEMBRANE protein transfer has been proposed to require information contained in a discrete portion of the protein molecule, called the signal sequence¹. The export of proteins to the cell envelope of the Gram-negative bacterium *Escherichia coli* seems to proceed, in most cases, by the use of signal sequences². Several examples have been found of proteins localised to either the outer membrane or the periplasmic space of this bacterium which are initially made as longer polypeptide chains containing signal sequences at their amino-termini. The preceding paper described the characterisation of mutants which prevent the secretion to the *E. coli* periplasm of the maltose binding protein³. In this paper, we focus on the transfer and localisation of an outer membrane protein, the bacteriophage λ receptor. This protein acts as the receptor on the cell surface for bacteriophage (ref. 4) and is also involved in the transport of maltose and maltodextrins^{5,6}.

The λ receptor protein is preferentially synthesised on membrane-bound polysomes⁷. In addition, it is initially synthesised in a larger precursor form^{7,8}. Recent studies involving DNA sequencing of the early part of the *lamB* gene (which codes for the λ receptor protein) and amino acid analysis of the λ receptor precursor synthesised *in vitro* have revealed that this protein has a 25-amino acid signal sequence at its amino-terminal end⁹.

We have devised a technique which has allowed us to select for mutations that prevent export of the λ receptor protein to the outer membrane¹⁰⁻¹². In such mutant strains, the unprocessed precursor form of this protein accumulates in the cytoplasm. We describe here the sequence analysis of the DNA of 15 of these mutants cloned into a bacteriophage λ vector. These mutants and those described in the preceding paper³ provide information concerning the role of the signal sequence in protein export.

DNA sequence of *lamB* gene in λ receptor export-defective mutants

The coding region for the amino-terminal 25-amino acid receptor signal sequence has been shown to be present on a 750-base pair *SalI* DNA restriction fragment⁹ (Fig. 1). The signal sequence is encoded by an early segment of this fragment. We have shown that 27 genetically and physically well characterised *lamB* mutations, which prevent export of the λ receptor pro-

tein, also map in this region¹².

We used the DNA sequencing method of Sanger *et al.*¹³. The well defined location of the mutations enabled us to select DNA primers that allowed sequencing of these mutations on both strands of the template DNA to ensure accuracy of the results. As shown in Fig. 1, the two primers chosen include a 67-base pair *HpaII-Hinfi* fragment and a 51-base pair *HpaII-HpaII* fragment. The 115-base pair region between these two primers contains the entire coding region for the λ receptor signal sequence⁹. Separated DNA strands of the *lapmalB13h80* phage containing these mutations were used as templates^{12,14} (see Fig. 1).

As previously described¹², the 27 *lamB* mutations affecting λ receptor export have been characterised as 14 point mutations and 13 deletion mutations. Detailed mapping of these independently isolated mutations has revealed that several of them are apparently repeats of the same mutational event. For this reason, only 15 of the 27 mutations were chosen for DNA sequencing. Eight point mutations and seven deletion mutations were sequenced.

The results (Fig. 2) of the sequences are: (1) all 15 mutations lead to alterations in the λ receptor signal sequence; (2) the eight point mutations represent single base changes in four distinct base pairs of the DNA. Three lead to a change in amino acid residue 15 (Ala to Glu), three in residue 19 (Met to Arg), one in residue 14 (Val to Asp) and one in residue 16 (Ala to Glu); (3) all the point mutations are transversions in the second position of each of four codons; (4) three of the deletions are internal to the portion of the DNA encoding the signal sequence. The remaining four deletions delete into this region of the DNA but are not confined to it.

Analysis of sequenced prokaryotic signal sequences

The DNA sequence of 15 mutations in the portion of the *lamB* gene encoding the λ receptor signal sequence has been determined. These mutations prevent export of the λ receptor protein to the outer membrane of *E. coli*, and instead, cause the accumulation of the precursor form of the λ receptor protein in the cytoplasm. These mutations and those described in the previous paper³ represent the first genetic proof of the functional role of the signal sequence in protein export or secretion. The nature of the amino acid alterations caused by these mutations indicates components of the signal sequence that are critical for the initiation of transmembrane protein transfer.

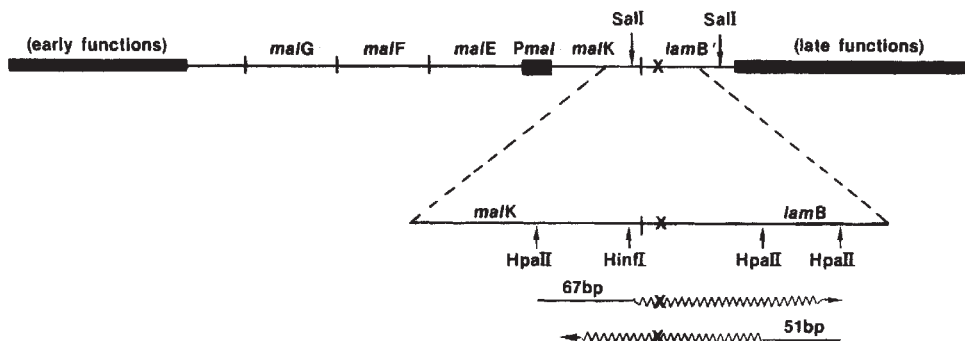
A comparison between known prokaryotic as well as eukaryotic signal sequences indicates that they share several similar characteristics¹⁵. The seven known sequenced prokary-

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Fig. 1 The λ malB13h80 phage contains the maltose transport genes, *malK*, *E*, *F*, *G* and approximately two-thirds of the *lamB* gene¹⁴. The putative λ receptor signal sequence mutations were recombined from the *lamB-lacZ* hybrid gene in which they were isolated into the portion of the *lamB* gene contained on this phage. The two critical *SalI* sites used in the physical mapping of these mutations are shown¹². To make template DNA for sequencing, phage DNA strands were separated on caesium chloride density gradients after heat denaturation in the presence of poly (U, G)^{9,25}. The two primers used in the sequencing were derived from plasmid pHCS (ref. 9). This plasmid carries a fragment of bacterial DNA which contains the entire *lamB* gene⁹. Plasmid DNA restriction fragments were separated on 4% polyacrylamide gels and were then electroeluted from the gel²⁶. The two primers, *HpaII-HinfI* and *HpaII-HpaII*, were used to prime on the separated *l* and *r* phage DNA strands, respectively.



otic signal sequences (Fig. 3) can all be broken down into two principal segments, a short amino-terminal hydrophilic basic segment followed by a predominantly hydrophobic segment of amino acids that extends up to the site of processing. There are from one to three basic amino acids (Arg and/or Lys) in the basic portion of the signal sequence. These positively charged residues have been suggested to play a part in initial attachment of the polysomes to the negatively charged inner surface of the cytoplasmic membrane^{15,16}. The hydrophobic segment directly follows the last basic residue in this initial segment. Charged amino acids, basic or acidic, are completely absent from this portion of the signal sequence. It has been suggested that this hydrophobic region loops into the membrane lipid bilayer or becomes associated with specific membrane protein(s), thereby initiating the transmembrane transfer of the polypeptide^{1,15-17}.

Possible mechanisms for the initiation of transport

The mutations described here for the λ receptor signal sequence and those described for the maltose binding protein (MBP)

signal sequence in the previous paper³ argue strongly for a critical role of the hydrophobic segment of the signal sequence in the initiation of the export process. The four different single amino acid changes in the λ receptor signal sequence and four of the five amino acid changes in the MBP signal sequence are all changes from hydrophobic (Leu, Met, Val, Ala) or weakly hydrophilic (Thr) amino acids to very hydrophilic charged amino acids (Arg, Lys, Glu, Asp), and all these changes occurred in the hydrophobic segment of the signal sequence. These mutations, therefore, strongly support the requirement of the hydrophobicity of this segment.

Note that a similar alteration in the lipoprotein (another outer membrane protein) signal sequence at amino acid position 14 also affects this hydrophobic segment³³. This mutation, which changes a glycine residue to an aspartic acid, does not have a dramatic effect on lipoprotein export, but does prevent processing of the lipoprotein precursor. The reason for this is not clear.

It is interesting that, in both the λ receptor and MBP systems, multiple copies of the same mutational events were isolated. In the λ receptor, at least, 17 of the 18 amino acid residues in the

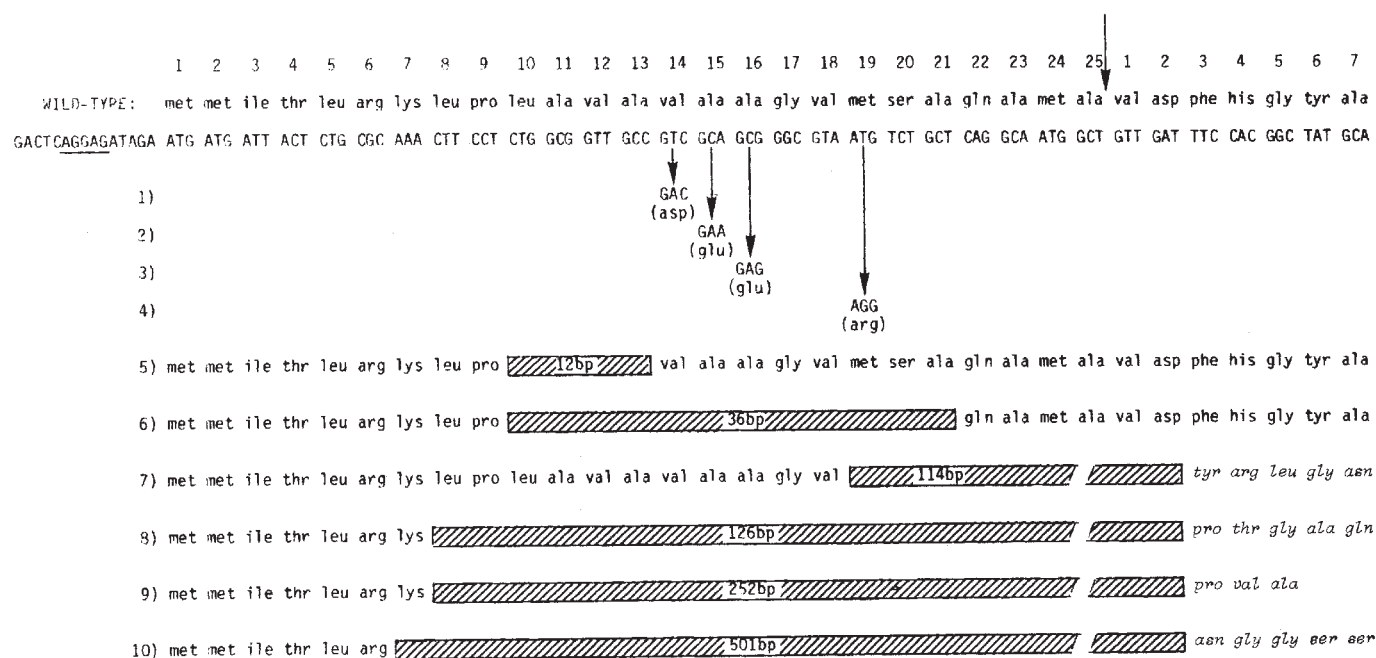


Fig. 2 Sequences shown were determined using the chain termination procedure¹³. Sequence reactions were run on thin 8% polyacrylamide gels²⁷. Gels were autoradiographed (Kodak No-screen X-ray film) for 12–24 h at –20 °C before reading off the above sequences. The signal sequence of wild-type λ receptor (top) and that of 15 *lamB* signal sequence mutations are shown. These mutations correspond to those present in the following *E. coli* strains: (1) SE2071; (2) SE2070, SE2079 and SE2105; (3) SE2099; (4) SE2069, SE2088 and SE2100; (5) SE2078; (6) SE2060 and SE2084; (7) SE2087; (8) SE2068; (9) SE2089; (10) SE2106. These strains are all described in detail in ref. 12. The amino acids to the right of deletions 7–10 above have not been confirmed by wild-type DNA sequencing. For this reason they are shown in italics. In (8), (9) and (10) above, the deletion end points are within codons. The amino acid directly following each of these deletions is, therefore, the result of a fused codon which may encode an amino acid not normally found at this position in the wild-type sequence.

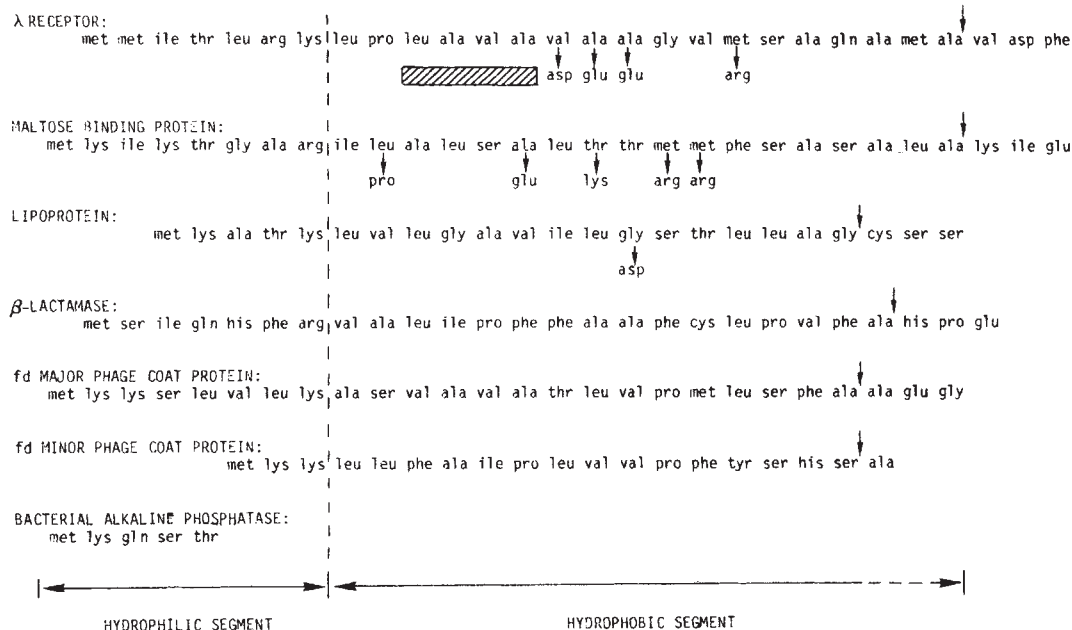


Fig. 3 The amino acid sequence of seven known prokaryotic signal sequences. These include λ receptor², MBP³, lipoprotein²⁸, β-lactamase²⁹, fd major phage coat protein³⁰, fd minor phage coat protein³¹ and alkaline phosphatase³². Mutational alterations in the λ receptor signal sequence, the MBP signal sequence³ and the lipoprotein signal sequence³³ are indicated below their corresponding wild-type sequences. The site of processing for these signal sequences is indicated above each sequence by an arrow.

hydrophobic portion of the signal sequence can be changed to charged amino acids by single base changes in the DNA. Despite this fact, of 14 independently isolated point mutations only 4 separate amino acids were changed. This suggests that only changes in a defined subset of the signal sequence amino acids will drastically affect export. (Although the *lamB* signal sequence mutants result in a λ-resistant phenotype, they are not totally defective in λ-receptor export, as small amounts are found in the outer membrane.) It may be that the lipoprotein mutation mentioned above has not affected such a critical amino acid. A more extensive mutant search will be necessary to confirm such a contention.

Export-defective mutations were not found in the amino-terminal basic segment of the signal sequence in either λ receptor or MBP. This might indicate that this portion of the signal sequence is not critical for protein export. Alternatively, the stringency of the selection for these mutations may not have allowed for detection of such mutants. The deletion and point mutations do, however, clearly demonstrate that this hydrophilic segment of the signal sequence is not sufficient for protein export.

The secondary structure of signal sequences has also been suggested to be important for the initiation of the secretion process¹⁶. Using available techniques for predicting polypeptide secondary structure^{18,19}, the structure of the λ receptor signal sequence and that of the mutants was determined. The results indicate that these mutations do not significantly alter the secondary structure of this signal sequence. Furthermore, the secondary structures of two signal sequences (for λ receptor and lipoprotein) seem to be quite different (unpublished results). However, the accuracy of these predictive techniques is questionable when considering such short peptides.

The fact that all the export-defective mutations studied lead to alterations in the signal sequence and not elsewhere in the proteins suggests that for these bacterial proteins the signal sequence is the only component of the protein required in the initial stages of translocation. Thus, the attachment of ribosomes to the cytoplasmic membrane and the initiation of the passage of such proteins through the membrane depend on an intact signal sequence. This agrees with synchronous translation experiments with the vesicular stomatitis virus glycoprotein which have shown that polysome binding to the membrane occurs at a time during translation when the signal sequence and little else of the polypeptide has been extruded from the ribosome²⁰. However, it seems likely that other components of the exported proteins

are essential in subsequent steps of the secretion process which ensure that such proteins arrive at their proper final destination. That this is the case is supported by recent studies on the export of certain hybrid proteins^{21,22}.

The results of the DNA sequencing have also indicated how at least some of the *lamB* deletions may have occurred. The 36-base pair deletions present in SE2060 and SE2084 represent two of six deletions that are genetically and physically identical¹². It is very likely that they are all the result of the same deletion event. Analysis of the DNA sequence in the region of the end points of this deletion reveals a segment of homology in direct repeat on either side of the deletion. There is evidence that limited homology of this type can promote specific deletion formation²³.

In genetic crosses of the λ receptor signal sequence mutants against *lamB*::Mu insertions, we found a very high percentage (18%) of the Mu insertions mapped under the 12-base pair deletion present in mutant SE2078¹². DNA sequencing of this deletion reveals no obvious reason for the apparent preference of Mu for this site. However, several of the sequences known to flank Mu insertions are present within the 12 deleted base pairs, including the most commonly occurring sequence, CAG²⁴. One can also detect DNA homologies in this region that would allow certain secondary structures in the DNA to form. The exact role these might have in Mu insertion is not clear. Continued analysis of new and other classes of apparent λ receptor signal sequence mutations should yield further insight into the mechanism of the important process of protein localisation.

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The λ phage *att* site: functional limits and interaction with Int protein

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*Site-specific integrative recombination of bacteriophage λ involves unequal partners. The minimal phage *att* site is composed of approximately 240-base pairs and four distinct binding sites for Int protein, at least three of which are crucial for function. This 'donor site' recombines efficiently with a smaller 'recipient site' that lacks the extensive interactions with Int protein.*

THE integration/excision pathway of bacteriophage λ is a particularly favourable system for studying site-specific recombination at the molecular level (for reviews see refs 1, 2). Integration of the phage genome into the chromosome of the host occurs by a reciprocal recombination between the phage *att* site (POP') and the bacterial *att* site (BOB'). The products are two prophage *att* sites which comprise the left (BOP') and right (POB') junctures between bacterial DNA and the integrated prophage (the formal equivalent of this recombination is shown in Fig. 1b). Both the integration and excision reactions require the phage-encoded protein Int as well as the products of several host *Escherichia coli* genes³. An additional requirement for the excision reaction is the phage-encoded protein Xis. The Int and Xis proteins are 356 and 72 amino acids respectively, as determined from their encoding DNA sequences⁴. Recent *in vivo* experiments suggest that in some situations, Xis can partially replace, or be replaced by, the host factors⁵. Highly purified Int protein forms specific stable complexes with *att* DNA, has an enzymatic activity for cutting and resealing DNA strands, and is functional in an *in vitro* intermolecular recombination system^{6–11}.

A structural feature of integrative recombination, both *in vivo* and *in vitro*, is that the phage *att* site must be on a negatively supercoiled DNA molecule^{8,11,12}. The cross-over event of Int-dependent recombination must take place within, or at the boundaries of, a 15-base pair core sequence that is common to all four *att* sites and is designated 'O' in the *att* site nomenclature¹³. DNA sequences of the four arms of the *att* sites, designated P, P', B and B', are all different from each other. The four *att* sites can also be distinguished from each other genetically by their distinctive behaviour in pairwise crosses^{14–16}.

The results reported here define the functional limits of the phage *att* site. We have also extended our studies of the interaction between Int protein and *att* sites⁹. The minimal phage *att* site is quite large (approximately 240 base pairs) and has several

widely spaced Int binding sites that differ in size and in response to heparin challenge. In contrast, the minimal sequence required for a phage *att* site partner (such as the bacterial *att* site) may not be much larger than the 15-base pair common core. We suggest a model in which integrative recombination involves two unequal partners; accordingly the phage *att* site is denoted the 'donor' and the bacterial *att* site or its analogue the 'recipient'.

Generating resected *att* sites

The λ phage *att* site is in the centre of a 492-base pair *HindIII*–*BamHI* restriction fragment that extends from positions –251 to +242. (For numbering DNA sequences in this region of the λ chromosome, the centre of the common core region is taken as 0; positive numbers extend to the right, through the *int* and *xis* genes, and negative numbers extend to the left¹³.) This *att*-containing restriction fragment was cloned into pBR322 in place of the *HindIII*–*BamHI* fragment from the *tet* gene. The resulting plasmid, pWR1, carries all of the information necessary to specify a functional phage *att* site as determined by its competence for Int-dependent recombination with a bacterial *att* site *in vitro* (see below). This plasmid was used as the starting point for defining the functional limits of the phage *att* site. The question is then, how much DNA can be removed from around the phage *att* site without impairing its function?

When comparing different plasmids for relative amounts of phage *att* site function it is desirable that only one of the two phage *att* site arms should be altered at a time, and that non-*att* sequences (that is, plasmid DNA) adjacent to the different shortened termini should all be the same. We therefore generated phage *att* sites with incremental lengths of one arm as summarised in Fig. 1. The first procedure involved exonucleolytic digestion of the terminal nucleotides and cloning of the shortened *att* sites. An additional procedure involved reassortment of shortened arms between two *att* sites by Int-dependent recombination and recovery of the products.

Protocols were designed so that the final *att*-containing restriction fragments always have one *HindIII* and one *BamHI*

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terminus, thus facilitating subsequent cloning, restriction analysis and sequencing. The details for the construction of each plasmid will be described elsewhere.

Function of resected phage *att* sites

Competence for phage *att* site function was tested in an *in vitro* Int-dependent recombination system^{10,11}. In the assay conditions used the phage *att* site must be on a supercoiled molecule¹¹ and the bacterial *att* site functions best when it is on a linear molecule (K. Mizuuchi, personal communication). Control experiments established that this assay registers only phage *att* site function on the supercoiled parent, and not prophage *att* site function (Fig. 3, right panel).

All plasmids carrying a P arm equal to or longer than that of pPH54 (–160) recombined efficiently with the bacterial *att* site, but none of those carrying a shorter arm (–115 or less) did so (Fig. 2 and Table 1). Thus some DNA between positions –160 and –115 is essential for the proper functioning of the P arm in a phage *att* site.

A family of P' arm-shortened plasmids generated as outlined in Fig. 1a, had a constant left terminus at –115. Because this did not include all the information required for a functional P arm (see above and Fig. 2), a longer P arm was restored to each plasmid as outlined in Fig. 1b. All *att* sites with P' arms equal to or greater than that of pPH307 (+82) were efficient recom-

bination partners, whereas those with shorter P' arms (+64 or less) failed to recombine efficiently (Table 1). The shortest P' arm that functions as a phage *att* site contains essentially all the DNA identified as forming heparin-resistant complexes with purified Int protein⁹. The longest P' arm which fails to function as a phage *att* site contains only half of this sequence.

To test whether the combination of a minimal P arm and a minimal P' arm is sufficient for full function, a phage *att* site carrying both minimal arms was constructed. A *HindIII*–*HinfI* fragment from –160 to –115 (from pPH54) and a *HinfI*–*BamHI* fragment from –115 to +82 (from pPH307) were cloned together into the *HindIII*–*BamHI* sites of pBR322. The resultant plasmid, pPH507 (–160 to +82), thus contains all the elements of a minimal P arm and a minimal P' arm, as defined by the experiments described above.

The function of this plasmid was compared with that of pPH7 (–115 to +82), pPH10 (–115 to +46) and pPH510 (–160 to +46). In recombination with the bacterial *att* site pPH507 recombined as well (60–100%) as the control plasmid pWR1, whereas the other three gave no detectable recombination (Fig. 3). In these assay conditions, supercoiled plasmid DNAs carrying the bacterial *att* site or the left or right prophage *att* sites all failed to recombine efficiently with the linear bacterial *att* site (Fig. 3). Therefore, the competence of pPH507 must reflect phage *att* site function (and not prophage or bacterial *att* site

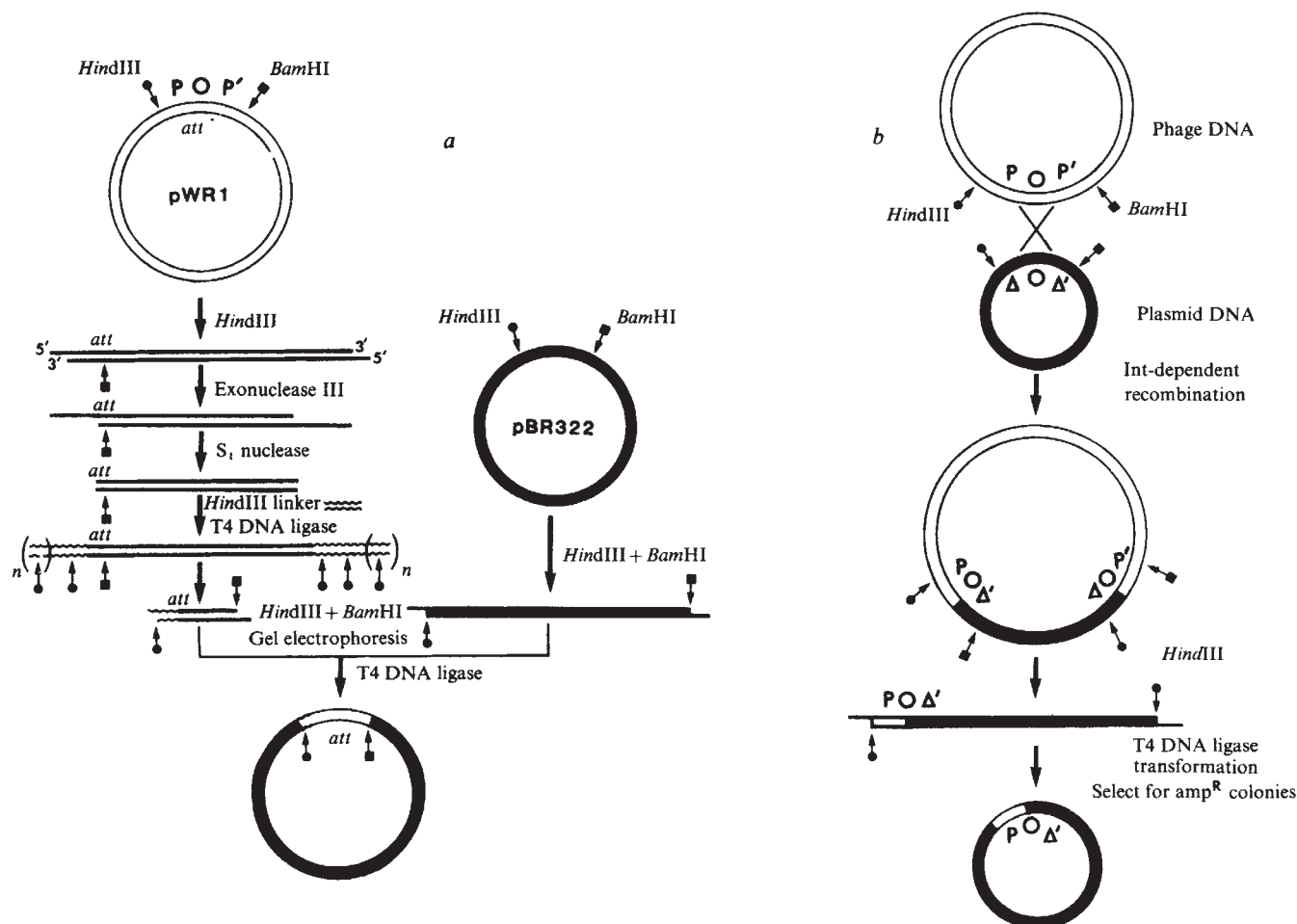


Fig. 1 Construction of plasmids carrying resected *att* sites. *a*, Plasmid pWR1 was first linearised, for example with *HindIII* for shortening the P arm. Digestion of the linear DNA with a combination of exonuclease III from *E. coli* (New England Biolabs) and nuclease *S*₁ (Miles) generates a series of shortened plasmids carrying flush base-paired termini. A chemically synthesised decamer *HindIII* linker (Collaborative Research) was ligated to the blunt end by T4 DNA ligase. Digestion with *HindIII* and *BamHI* generates a family of fragments with unaltered P' arms and P arms shortened to different extents. These were fractionated by gel electrophoresis and regions corresponding to the desired size were excised and eluted. Finally, the size selected fragments were recombined between the *HindIII* and *BamHI* sites of pBR322 and the plasmid carried in each clone characterised by restriction fragment analysis. In an analogous fashion, shortening of the P' arm would be carried out by using *BamHI* in the place of *HindIII* to linearise the plasmid and using a *BamHI* linker in place of the *HindIII* linker. *b*, Cells harbouring a plasmid with a shortened *att* site arm were infected with λ phage carrying a wild-type *att* site. As a result, some of the infecting phage underwent Int-dependent recombination with the *att* site on the plasmid and thus acquired the ability to transduce amp^R (D. Kamp and K. Mizuuchi, personal communication). The *att* site at each phage-plasmid junction has one arm derived from the plasmid and the other arm derived from the phage. Recovery of the recombinant *att* site on a self-replicating plasmid was accomplished by digesting the plasmid-phage hybrid DNA with *HindIII*, recircularising the DNA (by means of the *HindIII* ends) and selecting ampicillin-resistant transformants. These procedures will be described in detail elsewhere (P.-L. H. and A.L., in preparation).

Fig. 2 Integrative recombination of plasmids with resected P arms. Each reaction mixture contained ~1 µg supercoiled plasmid DNA with an intact, or P arm-resected, phage *att* site and 1 µg of linear DNA with a bacterial *att* site (*EcoRI*-*Bam*HI fragment; 1,600-base pairs) at a molar ratio of 1:3. The recombination was carried out in a 20-µl mixture containing 50 mM Tris-HCl (pH 7.4), 5 mM EDTA, 40 mM KCl, 12.5 mM spermidine, 4 µl of sonicated bacterial extract and 1.5 µl of Int fraction I (refs 8, 10). After incubation at 25 °C for 40 min, 30 µl of a buffer containing 0.3 M NaCl, 10 mM Tris-HCl (pH 7.4) was added and the samples were extracted with phenol-CHCl₃ before being loaded onto a 1% agarose slab gel and electrophoresed at 35 V for 16 h in E buffer (40 mM Tris, 20 mM Na-acetate, 2 mM EDTA, 18 mM NaCl, adjusted to pH 7.9 with acetic acid). The gel was stained with EtBr (1 µg ml⁻¹) for 15 min and then photographed under UV light. For quantitation the fragment containing the bacterial *att* site was first end-labelled with ³²P by T4 polynucleotide kinase⁹. After recombination and gel electrophoresis, portions of the gels corresponding to the unreacted linear DNA and the recombination product were excised and counted individually for ³²P radioactivities. The percentage of phage *att* site molecules that recombined with labelled bacterial *att* site DNA (present in threefold molar excess) was calculated as follows:

$$\% \text{ phage } att \text{ sites recombined} = \frac{^{32}\text{P radioactivity in the product band}}{\text{total } ^{32}\text{P radioactivity}} \times 3$$

The P arm end point of each resected plasmid, as determined by restriction enzyme analysis or by DNA sequencing is shown at the bottom (also see Table 1). All plasmids contain a P' arm extending to the *Bam*HI site at position +242. Positions on the electropherogram are indicated for the ³²P-labelled linear DNA containing the bacterial *att* site (*att*B), the unlabelled circular plasmid DNA containing the intact or resected phage *att* sites and migrating as supercoils (s.c.) or nicked circles (n.c.), and the linear ³²P-labelled products of recombination (arrows). Binding sites for Int protein (■) and the 15-base pair common core sequence (hatched) are indicated on the linear map of the *att* site region.

function). We conclude that the sequences delimited by the minimal P and P' arms (-160 to +82) are both necessary and sufficient for phage *att* site function.

Sequence and Int binding of minimal phage *att* site

Most of the DNA comprising the minimal region required for phage *att* site function was included in our reported sequence extending from -114 to +203 (ref. 13), within which two specific sites of Int interaction were detected⁹ (see below). One of these 30–35-base pair binding sites has at its centre the 15-base pair common core sequence where the cross-over occurs in recombination. The second site is in the P' arm (+50 to +86) and the sequence protected by Int extends to the right boundary of (3 to 4 bases beyond) the minimal length P' arm. Clones lacking part or all of this P' arm Int binding site failed to function as phage *att* sites (Fig. 3 and Table 1), indicating a requirement for this Int interaction for phage *att* site function (see below).

We now report the sequence for the remaining DNA in the P arm that is required for phage *att* site function as well as sequence extending further to the left (Fig. 4). The sequence from -250 to -200 is 47% A+T, in contrast to a relatively uniform distribution of 62% A+T from -200 to -115. When combined with the previously determined sequence¹³ this yields an average base composition of 71% A+T for the minimal phage *att* site.

The newly sequenced P arm region was examined for additional Int binding sites using the technique of DNase footprinting¹⁷. In the presence or absence of Int protein a singly end-labelled restriction fragment was digested with a nonspecific

nuclease in conditions where each molecule was cut approximately once. On a DNA sequencing gel the end-labelled fragments form a ladder, with gaps at those positions protected by Int. Using a restriction fragment that extends in both directions beyond the minimal phage *att* site, we observed that Int protects four regions from digestion by the AT-specific antitumour agent neocarzinostatin (Fig. 5a). Two of these regions correspond to

Table 1 Integrative recombination of plasmids with resected P arms or resected P' arms

Plasmid	End point		% Relative recombination†	
	P arm	P' arm	Expt 1	Expt 2
pWR1	-251	+242	100 (41)	100 (30)
pPH52*	-174	+242	48	100
pPH53	-160	+242	95	75
pPH54	-160	+242	99	106
pPH55	-115	+242	<0.5	<0.5
pPH56	-90	+242	<0.5	<0.5
pPH57*	-70	+242	<0.5	<0.5
pPH58*	-50	+242	<0.5	<0.5
pPH59*	-30	+242	<0.5	<0.5
pWR1	-251	+242	100 (32)	100 (20)
pPH303	-251	+124	113	80
pPH304*	-251	+100	95	75
pPH307	-251	+82	69	90
pPH308	-251	+64	<0.5	<0.5
pPH310	-251	+46	<0.5	<0.5

* The end points of these plasmids were determined by restriction fragment analysis. All other end points were determined by DNA sequencing.

† See Fig. 2 for quantitation. The results are normalised to 100% for pWR1. The absolute value for pWR1 in each experiment is shown in parentheses.

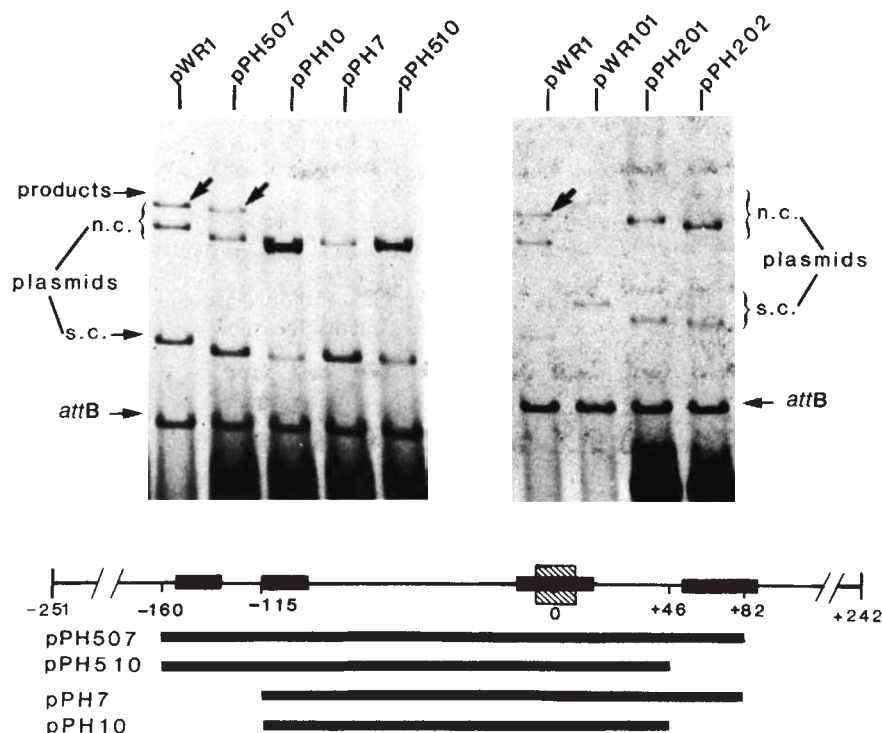


Fig. 3 Integrative recombination of the minimal phage *att* site. The *in vitro* recombination reaction and gel electrophoresis were carried out as described in Fig. 2 legend. The structure and boundaries of the cloned fragments used in the left panel were confirmed by restriction fragment analysis and by DNA sequencing. The extent of each cloned fragment is indicated by a long bar beneath the map of the *att* site region. The *att* sites carried by plasmids in the right panel are: pWR1-phage *att* site (*Hind*III-*Bam*HI-492), pWR101-bacterial *att* site (*Eco*RI-*Bam*HI-1,600), pPH201-left prophage *att* site (*Eco*RI-*Bam*HI-1,300) and pPH202-right prophage *att* site (*Hind*III-*Bam*HI-800). Positions on the electropherogram are indicated for the 'recipient' linear DNA containing the bacterial *att* site (*att*B), the 'donor' circular plasmid DNA containing truncated or intact phage *att* site and migrating as supercoils (s.c.) or nicked circles (n.c.) and the linear products of recombination (arrows). Binding sites for Int protein (■) and the 15-base pair common core sequence (hatched) are indicated on the linear map of the *att* site region.

the common core and P' arm binding sites⁹. The two others are located in the P arm, within the minimal phage *att* site; P arm site 1 extends from -148 to -129, and P arm site 2 from -116 to -98 (see also Fig. 4). Good agreement with these results was obtained by monitoring DNase I digestion of the complementary (bottom) strand (Figs 4 and 5b). These two binding sites are most probably the basis for nitrocellulose filter retention of Int complexed with P arm-containing restriction fragments¹⁸.

The importance of P arm site 1 is indicated by the fact that its removal abolished phage *att* site function (Figs 2, 3). However, it cannot be determined from exonuclease resection experiments whether P arm site 2 is also necessary. Base changes or deletions within P arm site 2 will be required to clarify this point.

An additional feature of Int-DNA interaction was visualised by DNase I, but not neocarzinostatin digestion. With Int concentrations higher than the minimum required to observe specific protection there was a series of strongly enhanced bands at 10-base pair intervals (Fig. 5b, lane 1, positions -75, -85,

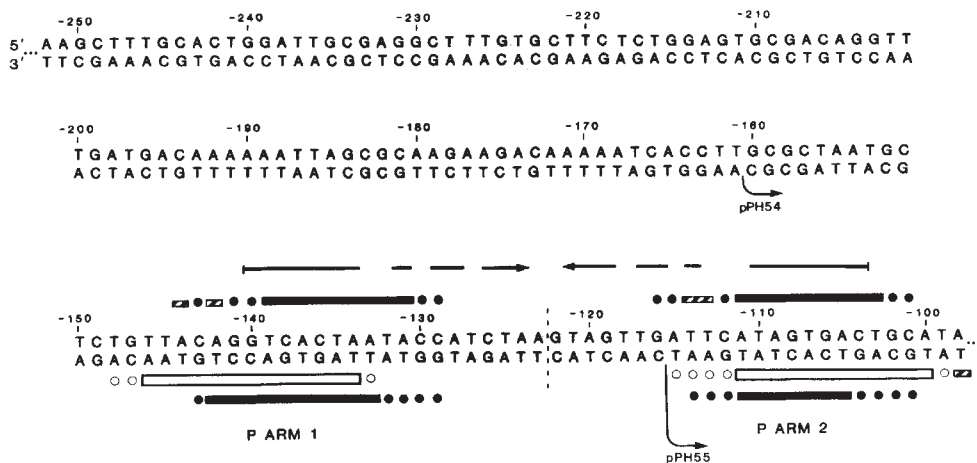
-95, -117 and -128). Similar patterns of enhanced DNase I cutting were also observed to varying extents on other restriction fragments in regions surrounding the common core and P' arm binding sites (data not shown), but not to the left of P arm site 1 (Fig. 5b). These results are to be distinguished from the possibly related observation that exonuclease digestion produced a 10-base pair pattern within the Int-protected sequence of the P' arm¹⁸.

Structural relations between Int binding sites

The P arm Int binding sites (15–20 base pairs) are each shorter than either the common core or the P' arm sites, but are comparable in size to the 15-base pair protected region in the *att*B common core⁹ (Fig. 6). However, the P arm sequences do not share homology with the common core sequence or with the core-arm junction sequences⁹.

The common core and P' arm Int binding sites show very little sequence homology with each other, possibly suggesting their

Fig. 4 Sequence of the left portion of the P arm and location of Int binding sites P arm 1 and P arm 2. P arm sequence is given negative numbers proceeding leftward from the centre of the common core region (0). We have previously reported the sequence from -114 to +203 (ref. 13). DNA sequence was determined for both strands (with the exception of -252 to -237) by the method of Maxam and Gilbert²⁷ using 5' termini labelled with ³²P by polynucleotide kinase²⁸; top strand sequence from -241 to -90 was obtained from the *Hind*III end of the *Hind*III (-251)-*Alu*(-6) fragment (see ref. 13); top strand sequence from -252 to -241 was determined using the *Eco*RI site of pBR322 in the plasmid carrying the *Hind*III(-251)-*Bam*HI(+242) *att* fragment (pWR1, see text). Complementary sequence, from -116 to -237, was obtained from the *Hin*I end of fragment *Hin*I(-115)-*Mn*I (recognition site at -231). The left boundaries of the shortest functional P arm (-160, pPH54) and the longest nonfunctional P arm (-115, pPH55) obtained in these experiments are indicated (↔). The locations of Int-binding sites P arm 1 and P arm 2 were determined by the method of DNase I footprinting¹⁷ with modifications described previously⁹ (see also Fig. 5). Int protection of sequences in the top strand from neocarzinostatin^{28,29} digestion (■) was carried out using two different fragments, each labelled at the *Hind*III end: *Hind*III(-251)-*Alu*(-6), carrying only P arm sequences, and *Hind*III(-251)-*Hpa*II(+305), carrying the entire minimal phage *att* site sequence (shown in Fig. 5a). Int protection of the bottom strand from neocarzinostatin (■) or DNase I (○) digestion was carried out on a fragment from plasmid pPH310 containing *att* sequence from +46 to -251 (*Hind*III), labelled at the *Bam*HI linker adjacent to position +46. Partially protected bases are designated ▨. Ambiguities in the precise boundaries of the protected regions are due to the failure of neocarzinostatin (●) or DNase I (○) to cut certain bases in the control digests. An inverted repeat structure (→ ←) which includes sequence from each of the two Int-binding sites plus additional sequence between the two sites is indicated.



interactions with different domains of the Int protein. In contrast, however, each of the P arm sites shares a considerable uninterrupted homology (11-base pairs in P arm 1 and 8-base pairs in P arm 2) with the left portion of the P' arm site. The two P arm sites also share this homologous sequence but with an inverted orientation, such that P arm 1 is a direct repeat and P arm 2 is an inverted repeat of the P' homology. A number of bases in the sequence shared among the P' and P arm protected regions have been identified as 'contact' sites in the Int-DNA interaction, as defined by methylation-protection experiments with the P' site (data not shown). Included within the left half of the P' site is the 7-base pair sequence, TCACTAT, which, in addition to being part of the homology with the two P arm sites, is found (with one mismatch) in the right half of the P' site.

Int binding in the P' arm has the unique property of resistance to challenge by the polyanion heparin (Fig. 5a and ref. 9). Furthermore, in studies with DNA carrying only the P' arm site (data not shown) we have established that the heparin-resistant binding is not dependent on the presence of the other binding sites. Thus, Int binding and resistance to heparin challenge are intrinsic features of the P' arm sequence. As Int binding at sequences homologous to the left half of the P' arm site (P arm sites 1 and 2) did not show the property of heparin resistance (Fig. 5a), the right half of this site, which is essential for recombination (Table 1), might be responsible for heparin resistance.

We have also used the footprinting assay to examine restriction fragments carrying other combinations of one to three Int binding sites (data not shown). The P arm 1 site was capable of binding Int when present singly. The common core region, when tested using a fragment containing att sequence from -115 to +46, bound Int independently of the P arm 1 and P' sites. Quantitative measurements to determine whether there are subtle differences in binding affinities as a function of the

simultaneous presence of two or more binding sites have not yet been carried out.

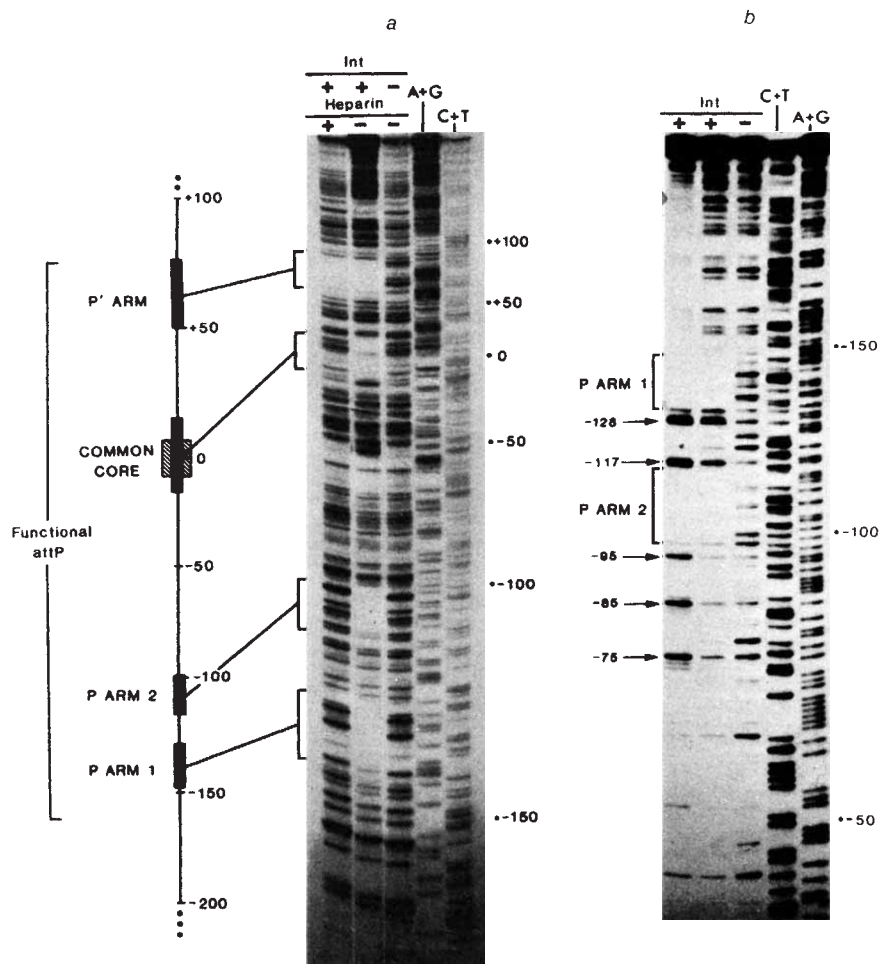
Efficient partners for phage att site

From *in vivo* Int-dependent crosses it was found that the phage att site recombines most efficiently with the bacterial att site and less efficiently with itself or either of the prophage att sites¹⁴⁻¹⁶. Similarly, in the *in vitro* conditions used here the phage att site recombined most efficiently with the bacterial att site. (Both *in vivo* and *in vitro* relative recombination values are strongly affected by reaction conditions and it is therefore difficult to make quantitative comparisons between them^{16,19-21}).

In our *in vitro* experiments the supercoiled phage att site recombined with the linear bacterial att site at a level of approximately 50% and with the linear right prophage att site (attR) at a level of approximately 5-10% (Fig. 7). With the linear left prophage att site (attL) the level of recombination was just above background in this experiment (not visible in the reproduced photograph), and recombination with the linear phage att site was not detectable. The relative recombination efficiencies were affected significantly by factors such as Int concentration, and this aspect of the reaction is being investigated. Nevertheless, we always found that a linear bacterial att site gave very high values and a linear phage att site gave very low values in their respective recombination patterns with supercoiled phage att site. Is this difference due to the presence of unique features that confer competence on the linear bacterial att site, or is it due to certain features on the linear phage att site (and linear attL) that inhibit recombination with a supercoiled phage att site?

Four plasmids containing the common core and different combinations of P or P' arm Int binding sites (as described in Fig. 3) were used as linear partners for a supercoiled phage att site (Fig. 7). It is clear from the fact that linear pPH10 recombines

Fig. 5 Footprints of Int binding on restriction fragments containing: *a*, the entire minimal phage att site (using neocarzinostatin); or *b*, the P arm and common core regions (using DNase I). *a*, In lanes 1-3 the restriction fragment HindIII(-251)-HpaII(+305), ³²P-labelled at the 5' end of the top strand (HindIII), was partially digested with neocarzinostatin^{28,29} in the absence (lane 3) or presence of purified Int protein at 10 $\mu\text{g ml}^{-1}$ (lane 2) or 20 $\mu\text{g ml}^{-1}$ (lane 1). The Int-DNA complex in lane 1 was challenged with heparin before neocarzinostatin digestion. Sequence markers for this restriction fragment, A+G (lane 4), and C+T (lane 5), were prepared according to the method of Maxam and Gilbert²⁷. Details of these methods and electrophoresis conditions have been described previously⁹. A linear map of the minimal phage att site region depicts the relative sizes and positions of the four Int protected sequences (■) seen in the neocarzinostatin footprint. Also indicated are the boundaries (-160 and +82) of the smallest functional attP region obtained in these experiments (see Fig. 3) and the 15-base pair common core sequence (hatched). *b*, In lanes 1-3 a restriction fragment from plasmid pPH310, containing att sequence from +46 to -251 (HindIII) (labelled at the 5' end of the bottom strand at the BamHI linker adjacent to position +46), was partially digested with DNase I in the absence (lane 3) or presence of purified Int protein at 2.5 $\mu\text{g ml}^{-1}$ (lane 2) or 5 $\mu\text{g ml}^{-1}$ (lane 1). The footprinting and electrophoresis conditions were as described previously⁹. The common core region has been run off this gel. Positions of enhanced cutting by DNase I (→) at ~10-base pair intervals (-75, -85, -95, -117, -128) are indicated in lane 1.



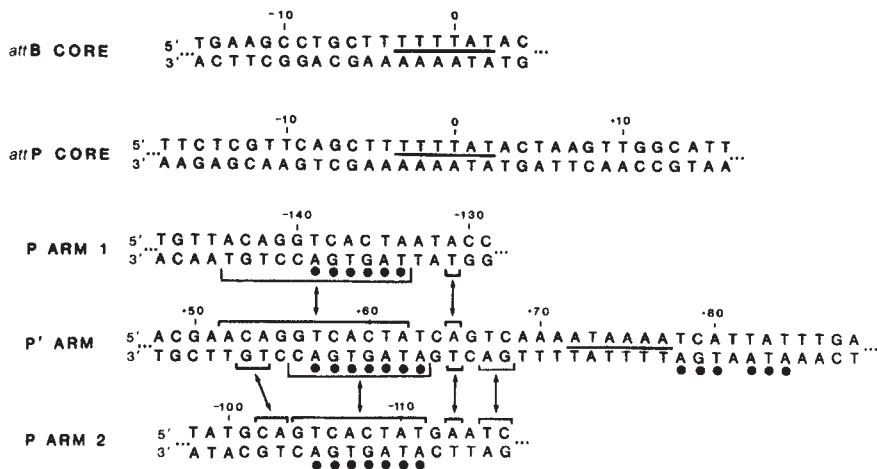


Fig. 6 Comparison of Int-protected sequences in the bacterial and phage *att* sites. The sequence shown for each binding site is the maximum length of DNA protected by Int from either neocarzinostatin or DNase I digestion (see Figs 4 and 5 and ref. 9). The sequence of the P arm 2 site has been inverted (compare Fig. 4) to facilitate comparison of sequence homologies. A 6-base pair homology between the two common core sites and the P' arm site is indicated by a line between the two strands of sequence (—). Homologies between the left portion of the P' arm site and each of the two P arm sites are indicated (—) (see text). A 7-base pair sequence in the left half of the P' arm protected region, found within the sequence shared with the two P arm sites, is also found, with one mismatch, in the right half of the P' arm site (●).

efficiently with supercoiled *attP* that an efficient partner for the phage *att* site requires neither the P arm 1 nor the P' binding sites. In this test of integrative recombination the bacterial *att* site does not possess any unique features that cannot be provided or mimicked by a phage *att* site lacking the P arm 1 and P' Int binding sites (compare lanes 2 and 6, Fig. 7).

The only difference between the two *att* sites that recombined efficiently with *attP* and the two that failed to recombine significantly was the presence of the P' arm Int binding site. Thus, the presence of the P' arm site on the linear partner was unnecessary, and actually interfered with its ability to recombine with a supercoiled phage *att* site. The P arm 1 binding site did not contribute as much interference (Fig. 7, compare pPH10 and pPH510). The interfering effect did not seem to be due to titration by the P' arm binding site as it was not abolished by higher concentrations of Int.

Discussion

Our results reveal the involvement in recombination function of approximately 240-base pairs of DNA adjoining the cross-over locus. Similar experiments, using a different method for obtaining shortened *att* sites, have been carried out by Mizuuchi and Mizuuchi²². In addition, they found function for an *att* site in which bacterial DNA is substituted for phage DNA up to position -152 (8-base pairs less than our shortest functional P arm, pPH54 and pPH507). Therefore, it is probable that a clone carrying phage *att* site DNA from positions -152 to +82 (235-base pairs) would also be functional.

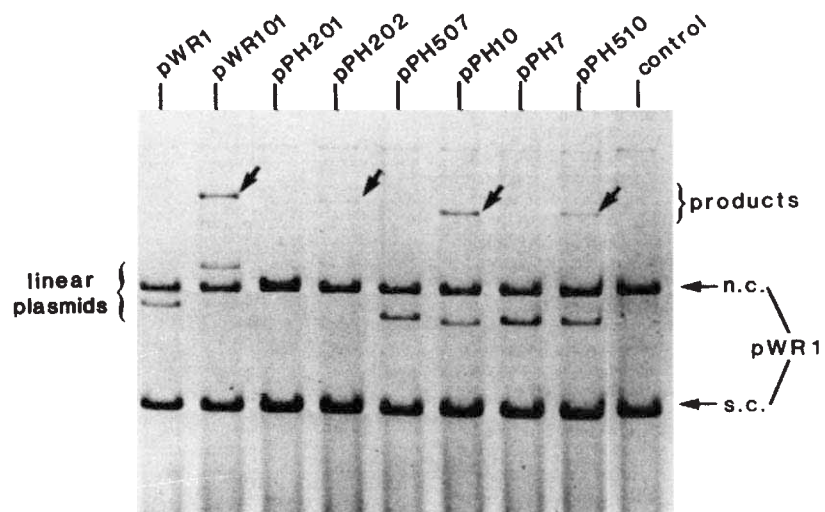
The diversity of the Int binding regions, both in sequence and in size (Fig. 6) is surprising. Considerable sequence homology is found among the three sites in the P and P' arms, but the sequence in the common core binding region does not share this homology. Sequence differences are almost certainly respon-

sible for the unique heparin-resistant character of binding in the P' arm site. The lack of homology between the core and the other sites could reflect more than one DNA-binding domain within the Int protein, or a single domain responding differently to the respective sequences. It is clear, however, that Int has at least two distinct capabilities. In addition to the 'structural' or DNA-binding properties involving sequences quite distant from the cross-over site of recombination, Int also has a nicking-closing (topoisomerase) activity¹⁰. Although no specificity for the phage *att* site has yet been demonstrated in the topoisomerase assays, it is clear from our results that Int does interact specifically with the normal substrate for this enzymatic activity⁹.

The nature of the role of the distant Int binding sites in the P and P' arms is much less apparent. Int binding seems to be an intrinsic feature of the individual DNA sequences since protection was seen for the P arm 1 and P' sites when present singly and for the common core in the absence of the P arm 1 and P' sites. The kinetics has not yet been studied, however, and it is possible that there is facilitation of binding at one site by Int bound at another site.

The two size classes of Int binding sites, approximately 30–35-base pairs and 15-base pairs, could reflect differences in the number of bound Int monomers. Similarities between the two P arm sites and each half of the larger P' site also suggest the possibility of higher order Int complexes. The linear distance between the P arm 1 and P' Int binding sites, or between the common core and either of these sites, is not incompatible with a three-dimensional structure in which a loop of DNA would bring any of these sites together in the same Int complex. For example, the distance between non-contiguous sequences which are in close proximity in the three-dimensional structure of the nucleosome is estimated to be approximately 80-base pairs²³. By analogy with studies on nucleosomes^{24,25} and DNA gyrase

Fig. 7 The efficiency of different *att* sites as linear recombination partners. The *in vitro* recombination reactions and gel electrophoresis were performed as described in Fig. 2 legend. Each reaction mixture contained supercoiled pWR1 (1.5 µg) and linear recipient DNA in a three to one molar ratio. The linear plasmids were generated by *Pst*I digestion of pPH7, 10, 507, 510 or by *Eco*RI digestion of pWR1, pWR101, pPH201 and pPH202. The origin and boundaries of the restriction fragments carried by each plasmid are described in Fig. 3 legend. Positions are indicated on the electropherogram for DNA containing different 'recipient' *att* sites (linear plasmid), the 'donor' circular plasmid DNA (pWR1) containing the phage *att* site and migrating as supercoils (s.c.) or nicked circles (n.c.) and the linear product of recombination (arrows).



complexes²⁶ the 10-base pair pattern of enhanced cutting around the binding sites (Fig. 5b) is suggestive of DNA wrapping around, or lying on, the surface of a higher order oligomer of Int. This idea is consistent with the electron microscope observation of Int complexes (8–10 monomers) interacting with the phage *att* site (Bob Yuan, personal communication).

Although their specific functions have not yet been defined, it is clear that the P arm 1 and P' Int binding sites have critical roles in recombination. Further experiments will be necessary to ascertain whether the P arm 2 binding site is also critical and whether the observed Int–DNA interactions are influenced by host factor proteins or by negatively supercoiled DNA substrate.

Our results suggest a model for integrative recombination that involves two unequal partners. The large size and complexity of the phage *att* site contrasts with the smaller size and simplicity of its partner. We refer to the phage *att* site, which must be supercoiled and which provides most of the structural information and specificity for the reaction, as the 'donor' molecule. A 'donor' *att* site recombines efficiently with an appropriate 'recipient' *att* site, such as the bacterial *att* site. Although we have yet to define the minimal requirements for 'recipient' *att* sites, several properties are known. We have shown previously that Int binding to the bacterial *att* site results in the protection

of a short (approximately 15-base pairs) DNA sequence at the left core–arm juncture⁹. The present results (and additional data, not shown) establish that a 'recipient' *att* site does not require the presence of the P arm 1 or P' arm binding sites. Furthermore, the absence of the P' site is necessary for a good 'recipient' *att* site. The sequence homology between the shortened phage *att* site (pPH10) and the bacterial *att* site, together with their respective Int protection patterns, suggests that an efficient 'recipient' site may not be much larger than the common core and partially homologous core–arm junctures¹³. Additional experiments will be necessary to confirm, and possibly extend, the applicability of this 'donor–recipient' model for integrative recombination.

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LETTERS

IR observations of the double quasar 0957 + 561 A, B and the intervening galaxy

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The properties of the remarkable double quasar 0957 + 561 were first described by Walsh *et al.*¹. Recently Young *et al.*² have described CCD observations of a distant galaxy associated with the quasar pair, and have identified this galaxy as a gravitational lens forming a double image of a single quasar. We report here 1.2–2.2- μ m observations of the system that support the conclusion that the twin quasars are a pair of images of a single object; the quasar has an energy distribution that is unusual. The intervening galaxy is shown to be highly luminous with a bolometric luminosity of about $2 \times 10^{11} L$.

As described by Walsh *et al.*¹ the system consists of two 17th magnitude visual objects with redshift $z = 1.4$; they refer to these as A and B, object A lying 5.7 arc s north of B. The galaxy

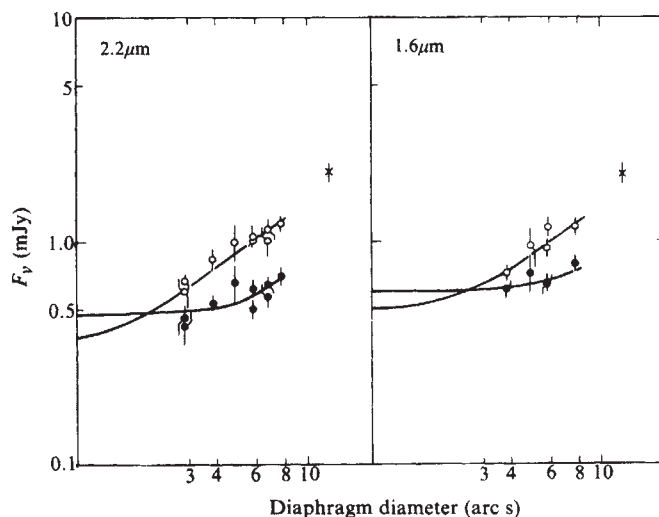


Fig. 1 The flux density plotted versus observing diaphragm for 1.6 and 2.2 μ m. ●, Observations centred on 0957 + 561A; ○, observations centred on 0957 + 561B. × indicates an observation made with a 12.5 arc s diaphragm centred between the two quasars. The solid lines are fits of a point source plus galaxy contribution in diaphragms centred on the quasars. The change in flux with diaphragm diameter for the galaxy is scaled from the visual observations of Young, *et al.*²

Table 1 Observed flux density of 0957+561 A, B

Diaphragm (arc s)	Date (UT) 1979	1.2 μm		Flux density (mJy) 1.6 μm		2.2 μm	
		A	B	A	B	A	B
2.9	17 December					0.45 $\pm$ 0.07	0.60 $\pm$ 0.09
	18 December					0.42 $\pm$ 0.08	0.66 $\pm$ 0.05
3.8	18 December			0.60 $\pm$ 0.05	0.71 $\pm$ 0.05	0.53 $\pm$ 0.05	0.89 $\pm$ 0.06
5.0	26 November*	0.6 $\pm$ 0.1	0.8 $\pm$ 0.1	0.72 $\pm$ 0.14	0.95 $\pm$ 0.19	0.66 $\pm$ 0.12	0.99 $\pm$ 0.18
5.8	18 December			0.65 $\pm$ 0.06	0.94 $\pm$ 0.08	0.62 $\pm$ 0.06	1.01 $\pm$ 0.08
	2 November			0.64 $\pm$ 0.06	1.16 $\pm$ 0.12	0.51 $\pm$ 0.05	1.06 $\pm$ 0.11
6.7	17 December					0.57 $\pm$ 0.08	1.00 $\pm$ 0.15
						0.64 $\pm$ 0.05	1.13 $\pm$ 0.11
7.7	18 December			0.79 $\pm$ 0.06	1.17 $\pm$ 0.08	0.70 $\pm$ 0.07	1.21 $\pm$ 0.09
12.5	17 December	1.2 $\pm$ 0.3		2.1 $\pm$ 0.2		2.1 $\pm$ 0.2	

* At 5-m Hale telescope.

found by Young *et al.*² lies 0.8 arc s north of B; it has a redshift of 0.4 and has angular dimensions of the order of 5 arc s.

The observations reported here consist of IR photometry of the twin quasars at 1.6 and 2.2 μm through a series of diaphragms. These observations were obtained using the 3-m telescope of the NASA IR Telescope Facility at the Mauna Kea Observatory and the 5-m Hale telescope on Palomar Mountain. The most extensive observations were obtained on the nights of December 16 and 17 1979 at Mount Kea in conditions when the seeing disk was ≤ 1 arc s. Variations in seeing during the multiple-diaphragm observations were accounted for by observing the star ϕ Ursae Majoris as a secondary standard before each observation of the quasar pair. This sequence was repeated for each wavelength and each diaphragm used. In addition to the multiple diaphragm observations on each quasar, observations were obtained at 1.2, 1.6 and 2.2 μm with a 12.5 arc s diaphragm centred between the two quasars.

Table 1 and Fig. 1 show the observed flux densities within various diaphragms centred on quasars A and B. The 1.6- and 2.2- μm observations of quasar B show a strong dependence of flux on observing diaphragm, the observed 2.2- μm flux doubling between the 3 and 8 arc s diaphragms. This observation clearly shows the presence of extended IR emission in the vicinity of quasar B. A similar, but much smaller, effect is also seen in the observations of quasar A.

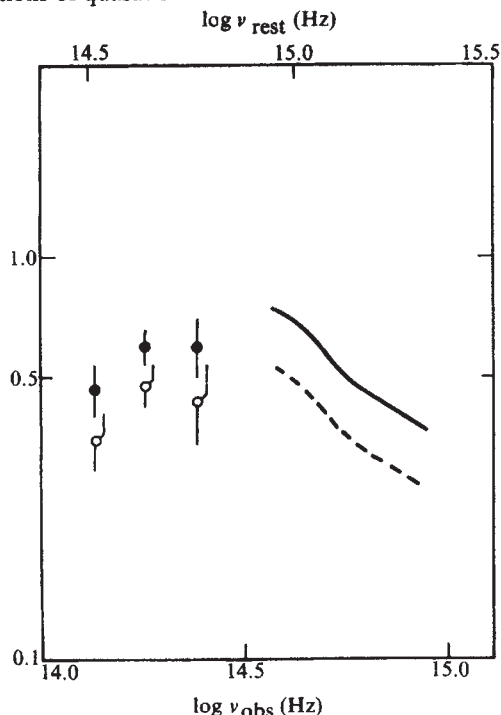


Fig. 2 The energy distributions of the quasars 0957+561A (●) and B (○) from 0.35 to 2.2 μm . The data have been corrected for the contribution of the underlying galaxy and represent only the quasar contributions. At visual wavelengths, the flux densities for quasar B are taken as 0.72 of quasar A, as per Young *et al.*².

We interpret the extended emission as being due to the galaxy identified by Young *et al.*². To separate the contributions to the multiple-diaphragm observations from the galaxy and from the quasars the light distribution for the galaxy, as reported by Young *et al.*², has been numerically integrated for various diaphragms centred on quasars A and B. The IR observations centred on quasar B were first fitted as the sum of a quasar component which is constant with diaphragm plus a galaxy component for which the IR flux density was assumed to follow the visual light distribution as measured by Young *et al.*². The best fit to the observations centred on B is included in Fig. 1 and listed in Table 2; for this fit, 42% of the 2.2- μm flux density in a 3-arc s diameter diaphragm arises in the intervening galaxy. To determine the flux densities of quasar A, the galaxy contribution to the observations centred on A was determined from the fit to the observations centred on B. The best fit to these observations is also shown in Fig. 1.

The ratio of fluxes of the B and A quasars are $F_\nu(B)/F_\nu(A) = 0.80 \pm 0.15$ and 0.74 ± 0.15 at 1.65 μm and 2.2 μm respectively. These ratios are consistent with the ratio of B/A found visually by Young *et al.*² of 0.75 ± 0.04 and at radio wavelengths of 0.80 ± 0.05 (refs 3, 4). Thus, in all observed wavelength ranges, the two quasars have a constant flux ratio, consistent with the achromatic imaging predicted by the gravitational lens model. This conclusion is valid if there is no significant difference in the extinction along the two separate light paths through the intervening galaxy.

The 0.35–2.2- μm continuum distribution of the quasars shown in Fig. 2 is unusual. The increase in flux density from $10^{14.5}$ to 10^{15} Hz in the quasar rest frame is rare; of the 39 quasars observed by G.N. *et al.*⁵ all but one show a negative spectral index at these wavelengths. The similar energy distribution of quasars A and B make them far more similar to each other than to any other known quasar. This again strongly suggests the idea that these objects are two images of a single physical object, rather than two quasars in the same cluster.

Because the light emitted from the galaxy dominates the flux longward of 1 μm , the properties of the galaxy can be inferred from a fit to the IR observations. Figure 3 shows the energy distribution of the galaxy in a 7-arc s diaphragm; Table 2 gives the flux densities of the whole galaxy at 1.2, 1.6 and 2.2 μm . Young *et al.*² find an R magnitude for the entire galaxy of $R \sim 18.5 \pm 0.2$ mag; thus $(R - [2.2])_{\text{galaxy}} = 4.3 \pm 0.3$ mag and $([1.6] - [2.2])_{\text{galaxy}} = 0.8 \pm 0.3$ mag. These colours are consistent with those found for luminous galaxies at a redshift of around 0.4 by S. E. Persson, K.M. and G.N. (unpublished results).

Table 2 Derived flux densities

	1.2 μm *	1.65 μm	2.2 μm
0957+561 A	0.6 $\pm$ 0.1	0.60 $\pm$ 0.06	0.47 $\pm$ 0.07
0957+561 B	0.4 $\pm$ 0.1	0.48 $\pm$ 0.06	0.35 $\pm$ 0.06
Galaxy (12.5 arc s)	0.6 $\pm$ 0.3	0.95 $\pm$ 0.15	1.23 $\pm$ 0.15

* The flux densities derived at 1.2 μm are based on extrapolations and have unknown systematic uncertainties.

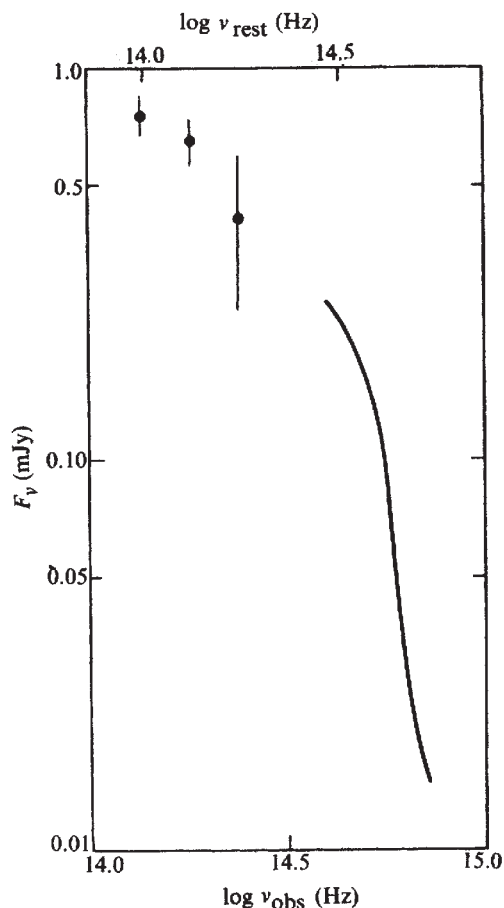


Fig. 3 The energy distribution from 0.35 to 2.2 μm of the galaxy associated with the quasars 0957+561A, B as derived from observations in a 7 arc s diaphragm centred on the B quasar. At all wavelengths, the contribution from the quasars have been subtracted from the observed flux to yield this energy distribution. At 1.2 μm the equivalent flux density in a 7 arc s diaphragm has been derived from observations in a 5 arc s diaphragm. The visual observations are from Young *et al.*²

If the Hubble constant $H_0 = 60 \text{ km s}^{-1} \text{ Mpc}^{-1}$ and the deceleration parameter $q_0 = 1$, the luminosity of the galaxy in the observed wavelength range 0.4–2.2 μm is $2 \times 10^{11} L_\odot$, establishing it as a highly luminous galaxy. If the energy distribution of this galaxy resembles that of the giant elliptical galaxy M87, which has a bolometric luminosity of $1.3 \times 10^{11} L_\odot$ (ref. 6), then the bolometric luminosity of the lens galaxy in the double quasar system is $2.4 \times 10^{11} L_\odot$.

In conclusion, IR observations of the double quasar 0957+561 A,B support the gravitational lens model of these objects in two significant ways.

(1) The ratio of the IR flux densities of the two components is the same as found in the visual and at radio wavelengths. Furthermore, the derived energy distributions for the two components are the same and extremely unusual. This strongly supports the suggestion that quasars A and B are indeed two images of the same physical object.

(2) The IR properties of the lens galaxy are entirely consistent with it being a giant elliptical galaxy at a redshift of ~ 0.40 .

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Spectra of interplanetary scintillation

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A new type of radio spectrograph is described here which has been used to observe the interplanetary scintillation of a radio source over a two-to-one frequency range. Simultaneous observations over this range provide a direct insight into the time variations of intensity and the physical form of the interplanetary irregularities producing them. A specific example shows the direct way in which the spectral record can indicate the physical details of the electron density variations of the interplanetary medium.

The scintillation of small-diameter radio sources¹ due to scattering in the outer layers of the solar corona has been analysed statistically^{2,3} and the observations fit the theory of diffraction at a thin screen reasonably well⁴. The corona is subject to strong day-to-day variations, and some of the inconsistencies would be due to the non-simultaneous observations at the specific frequencies used in these comparisons. However, the theories are not complete and particular problem areas include^{3,5} the actual form of the irregularities, and an excess of enhanced emission occurring as the scattering moves from being weak to strong—the r.m.s. phase deviation on the wavefront emerging from the screen increases to 1 rad and beyond. It is in these areas that the spectral observations are particularly illuminating.

The observations are made with a new and unique form of radio spectrum analyser, the acousto-optical radio spectrograph. The broadband radio signal from the radio telescope is converted to a travelling ultrasonic wave in a solid medium. A Bragg interaction between the travelling ultrasonic beam and a transverse, collimated beam from a helium-neon laser produces diffracted light whose angular intensity distribution can be shown to be the power spectrum of the applied radio signal^{8,9}. In the observations discussed here, the Parkes 64-m radio telescope was used with a broadband feed and amplifier in such a way that the frequency range 280–520 MHz was analysed by the spectrograph. The optical output was detected by a self-scanned array of photodiodes and digitized^{6,7}. The 240 MHz bandwidth was resolved into 256 simultaneous channels and complete spectra were recorded on magnetic tape 25 times per second.

Source 3C273 was observed on 10 and 11 October 1978, when its line of sight was 13° (0.23 AU) from the Sun's centre. At this position the current theory⁴ predicts that the mean r.m.s. phase deviation, ϕ_0 , varies from ~ 0.65 to ~ 0.35 rad over 280–520 MHz respectively and that the scale size, a_0 , of irregularities, assumed to have a correlation distribution $\exp(-r^2/2a_0^2)$, is ~ 55 km. If one defines a Fresnel distance z_0 as $2\pi a_0^2/\lambda$, these values of a_0 correspond to z_0 varying between 0.12 and 0.25 AU over 280–520 MHz respectively. On an average day one would be well into the 'far-field' of the diffraction. A sample of the data obtained on 11 October is shown in Fig. 1 with a 40-ms time resolution and smoothed to 53 frequency channels over the range 280–520 MHz. Without resorting to statistical analysis one can see that the variations are highly correlated over the frequency range and are dominated by both weak and strong curved ridges of emission which take about 1 s to sweep across the frequency range.

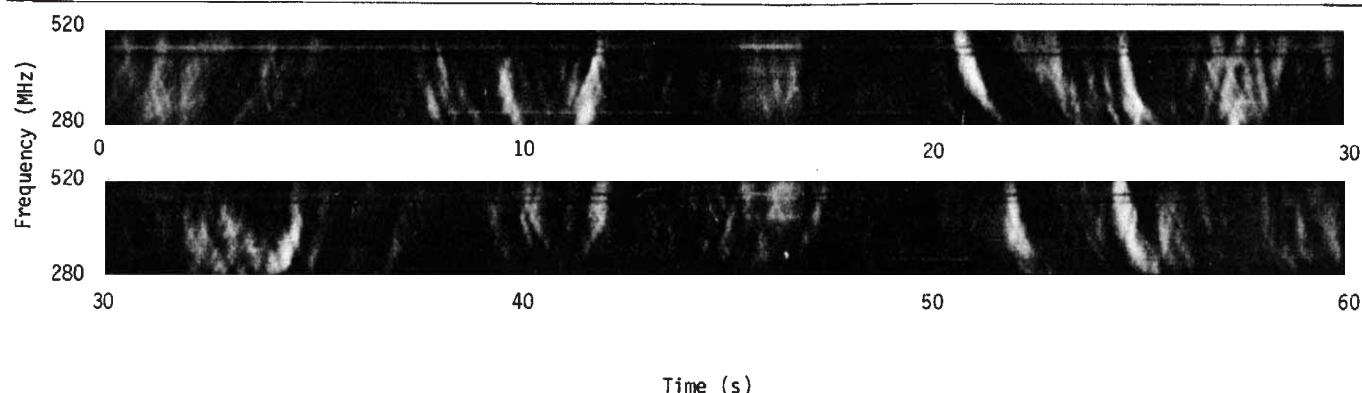


Fig. 1 An example of the intensity variations of source 3C273 due to interplanetary scintillation. Systematic structure on the frequency-time plane is evident. The figure is plotted with a frequency resolution of 4 MHz, time resolution of 40 ms, and white represents increased intensity.

As suggested by Hewish, the simplest explanation for the curved bands in Fig. 1 is that a normal, random diffraction pattern has been refracted by large wedge-shaped irregularities which are themselves too large to produce intensity variations. This would account for the increasing curvature at lower frequencies, where the refractive index of the plasma is also higher.

However, the dynamic spectrum reveals details which cast doubt on such an explanation. It can be seen that Fig. 1 contains bands of a characteristic shape. That is, the curvature at any frequency is always of essentially the same magnitude although the sign of the curvature can vary. The refracting wedges would need to be of constant wedge angle. The wedge would also need to change sharply from this angle to the negative value of the same angle within 1 s to explain sections of Fig. 1 such as at 10 s and 41 s. An even greater difficulty is illustrated by Fig. 2, where the section of Fig. 1 at around 45 s is shown in greater contrast. As at other parts of the record, bands of opposite sign are observed superimposed. Multiple refracting wedges in the line of sight would simply add the refracting angles algebraically and one could not expect to maintain the characteristic curvatures observed.

Further details are revealed in the more intense bands, one of which is displayed in higher contrast in Fig. 3. The peak intensity takes about 1 s to sweep across the frequency range. At an assumed solar wind speed of 400 km s^{-1} , this represents an angular displacement of 400 km in 1 AU or $0.55''$. If the refractive index (and hence refracting angle) of the plasma varies as f^{-2} , then it is 3.5 times greater at 280 than at 520 MHz. The $0.55''$ then represents the difference in refraction angles or 2.5 times the refraction angle at 520 MHz. The increasing curvature to the band indicates a typical refraction angle of $0.22''$ at 520 MHz and $0.77''$ at 280 MHz.

Figure 3 also indicates a feature visible on all the stronger bands of Fig. 1. At the higher-frequency end and always on the

inside of the curvature is a region of intensity which is reduced below the mean intensity level. This is also indicative of refraction if the figure is interpreted as interference between a plane wave and a wave impinging at an angle. The strong band is a region of constructive interference and the reduced intensity is where a half-wavelength phase difference produces destructive interference. These successive interference fringes are seen to be about 0.6 s apart in time at 520 MHz or 240 km for an assumed solar wind speed of 400 km s^{-1} . The corresponding angle at 520 MHz is a half-wavelength in 240 km or $0.25''$. Both the curvature and fringes indicate the origin for the band as

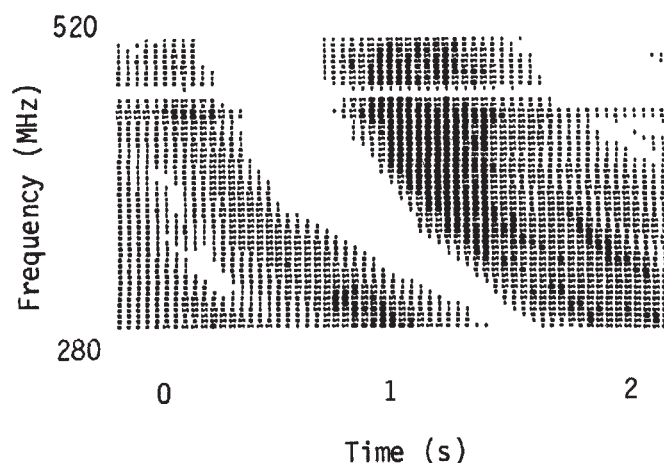


Fig. 3 A high-contrast picture of a strong band reveals the characteristic increasing curvature and the successive bright and dark interference fringes at the higher frequencies.

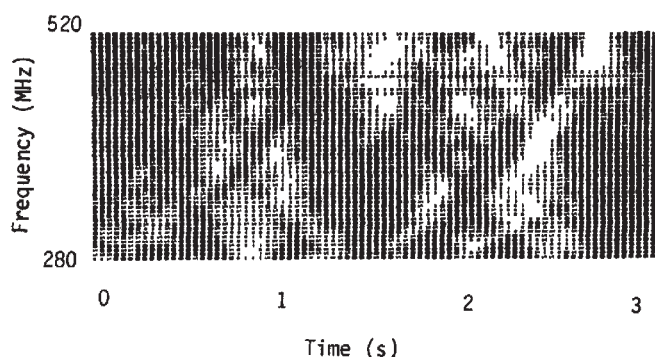


Fig. 2 A section of Fig. 1 is reproduced with increased contrast to illustrate the superposition of bands of opposite curvature.

being due to refraction. The interference fringes are, however, also incompatible with the refraction being due to a large wedge. With a large wedge the background wave would also be refracted and the interference would not be observed.

An alternative explanation is that the bands are the result of individual refracting elements in the interplanetary medium. That is, at least for this solar elongation, there are insufficient irregularities in the line of sight to produce a random diffraction pattern. A picture emerges of irregularities in the form of comparatively sharp gradients in the plasma density. This picture is quite different from that usually assumed and certainly different from the 'weakly scattering' assumptions at the basis of work such as that of Uscinski³. The observed fact that strong, individual bands or 'spikes' are seen only at the smaller solar elongations⁵ is also consistent with one seeing fewer irregularities in the line of sight and, at least for the stronger cases, the effects of individual irregularities.

More observations, analysis and modelling are to be done, but even these preliminary results suggest the value of dynamic spectral observations in the analysis of interplanetary scintillations. Just as spectral observations of scintillations from the ionosphere gave greater insight⁸ than single-frequency studies, so the spectral observations reported here extend single-frequency interplanetary studies. The fact that single-frequency observations of interplanetary scintillations might obey Rayleigh statistics is not always sufficient proof that a random scattering medium is actually involved. One possibility suggested here is of systematic diffraction from sharp density gradients.

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An alternative interpretation by A. Hewish*

Cole and Slee have suggested that their beautiful observations of interplanetary scintillation cannot be explained by conventional models of the solar plasma. I do not share their view and now outline my reasons for believing that the new data reinforce the standard theory.

On conventional models, derived from spacecraft and radioastronomical data, the solar plasma contains density irregularities having a three-dimensional wavenumber power spectrum $P(K)$ which approximates to a Kolmogorov spectrum $P(K) \propto K^{-11/3}$ for irregularities on a scale $10^6 \text{ km} > 2\pi K^{-1} > 10^3 \text{ km}$. Evidence for a flattening of the spectrum for $2\pi K^{-1} < 10^3 \text{ km}$ has been reviewed elsewhere^{1,2}. At a solar elongation of 13° , and at 400 MHz, Readhead *et al.*¹ predict intensity fluctuations on a time scale of about 0.7 s, with a scintillation index of about 0.75, and which correlate over a band of about 200 MHz. These features are in good agreement with Fig. 1.

It is well known that irregularities on a scale larger than about 10^3 km (at this elongation) give reduced intensity fluctuations at 400 MHz because the Earth is then within the near-field zone. The larger irregularities, therefore, introduce phase gradients across the incident wavefront. I have shown elsewhere³, in connection with the interstellar scintillation of pulsars, that scintillation due to small-scale irregularities combined with phase gradients due to large-scale irregularities produces characteristic frequency drifts similar to those observed by Cole and Slee. The drift rate is

$$\frac{d\nu}{dt} = \pm \frac{V\nu}{2Z\theta_r}$$

where V is the drift velocity of the diffraction pattern, Z the distance to the irregularities, and θ_r the angular refraction corresponding to the phase-gradient. This phenomenon was originally discussed by Shishov⁴.

The observations of Cole and Slee show frequency drifts which change sign in about 5 s, corresponding to a periodicity of roughly 10 s. This requires irregularities of scale $2\pi K^{-1} \sim 4 \times 10^3 \text{ km}$ assuming a solar wind speed of 400 km s^{-1} . The model of Readhead *et al.*¹ indicates a phase modulation $\phi \sim 0.53 \text{ rad}$ at 400 MHz for small-scale irregularities which produce scintillation on a time scale of about 0.7 s. Assuming $P(K) \propto K^{-11/3}$

we derive $\phi \sim 0.53(10/0.7)^{5/6} \approx 5 \text{ rad}$ for the integrated phase modulation on scales up to $4 \times 10^3 \text{ km}$. Putting $\theta_r \sim \phi K\lambda/2\pi \approx 0.2 \text{ arc s}$ we obtain $d\nu/dt \sim \pm 390 \text{ MHz s}^{-1}$ for $Z = 1 \text{ AU}$ and $\nu = 400 \text{ MHz}$. This drift rate is typical of many features seen in Fig. 1. The question of simultaneous drifts with opposite sign mentioned by Cole and Slee raises no problems. The magnitude of θ_r ensures that wave fronts refracted convergently at a distance of 1 AU must, on arrival at the Earth, be overlapping for $\sim 10\%$ of the total time, leading to the superposition of patterns having drifts in opposite directions. The convergence and divergence of wave fronts suggest that overlap regions should be flanked by bands of negative slope on the preceding side, and positive slope on the following side, which is certainly true for the feature at 45 s discussed by Cole and Slee. They should also be associated with a higher mean intensity which can again be seen.

Finally it should be mentioned that the 'spiky' nature of the intensity variations is typical of diffraction patterns at distances not too far from the 'focusing' distance of the random irregularities. This property has been reviewed by Uscinski⁵. I conclude that the standard theory, involving the integrated effect of many 'weak' irregularities along the line of sight, is in excellent agreement with the present observations. I believe that more extended data of this kind could add considerably to our knowledge of $P(K)$.

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Crystal structure of Ag · TCNQ

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High resolution electron microscopy (HREM) has been used for the direct imaging of complex crystal structures. High resolution at an atomic level cannot be expected from most organic crystals due to their high sensitivity to electron radiation damage, although we¹ have previously considered the conspicuous images of chlorine and copper atoms in chlorinated copper phthalocyanine molecules as a special case dependent on the very radiation-resistant property of the compound. In practice, however, some organic crystals may be able to withstand the dosage necessary for the image recording itself but could not tolerate the extra dosage necessary for searching the proper image field and for successive careful focusing. In this respect, the principle of the minimum exposure proposed by Williams and Fisher² is attractive as it might reduce the unnecessary damage which takes place before the actual image recording. We describe here the structure images of a metal complex of 7,7',8,8'-tetracyanoquinodimethane (TCNQ) with the resolution of an atomic level obtained by developing an automated device which is basically similar to that by Unwin and Henderson³. The electron microscope equipped with the low dosage device was JEM-200CX, which gives the instrumental resolution of 0.24 nm.

The various metal salts of TCNQ behave as the charge transfer complexes which show interesting electronic and magnetic properties. A thin crystalline film of complex salt forms by solid state diffusion when a thin film is prepared in the form of a double layer by the successive vacuum condensation of TCNQ and metal on a suitable substrate⁴. If the metal film is deposited on TCNQ crystallites which have been epitaxially grown in advance on a KCl cleavage face, the reaction leads to a

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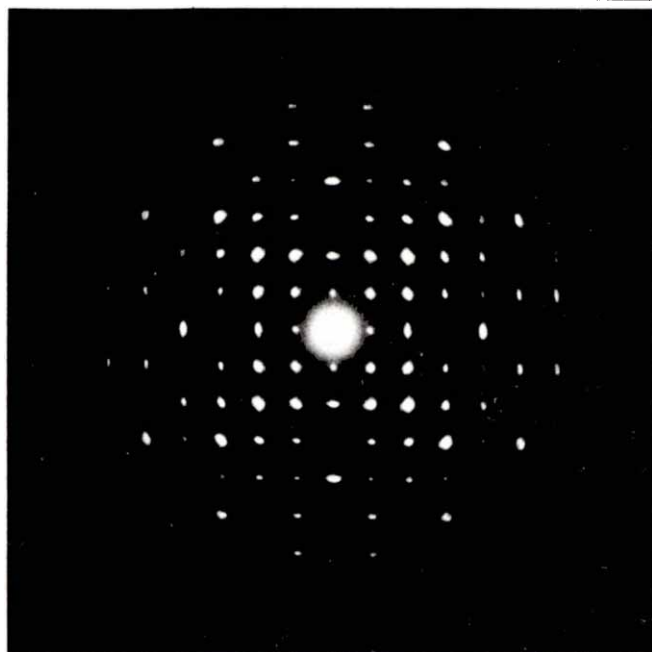


Fig. 1 SAD pattern from a crystallite of Ag·TCNQ salt taken with the incident electron beam parallel to the *c*-axis of a tetragonal unit cell.

topotactic formation of the complex crystallites which eventually assume characteristic orientations. This type of solid state formation of complex salts was found to be possible in general with many metals such as Al, Ag, Au, Co, Cs, Cu, Fe, Li, Mn, Ni, Ti and Zn. In the present work, we used very thin crystallites of Ag·TCNQ because the medium heavy ions such as silver were expected to give sufficient contrast for the clear recognition of their individual images. The selected area diffraction (SAD) indicated that the crystal assumes a tetragonal unit cell the dimensions of which are $a = 1.25$ nm and $c = 0.70$ nm. With the electron beam incident normal to the specimen habit face, the single crystal pattern presents a characteristic tetragonal symmetry as shown in Fig. 1 and the reflections are expected to contribute to the formation of high resolution images. To avoid the confusing effect of the dynamical electron scattering on the image formation, the crystal thickness was carefully controlled to be as thin as 3 nm by monitoring it with a quartz crystal microbalance.

The images of Ag·TCNQ complex salt were recorded on Fuji FG films for 0.7 s at the electron optical magnification of 170,000 with the electron dosage of ~ 0.2 C cm $^{-2}$. The optical density of the film developed with Kodak D-19 was < 0.5 , causing a considerable amount of photographic noise which was enhanced by the quantum fluctuations in the images. To increase the signal-to-noise ratio the final images were translationally averaged over the crystal periodicities along the two tetragonal axes by applying the photographic multiple exposure technique. An enlarged photomicrograph of the structure image thus obtained is reproduced in Fig. 2a.

The characteristic features of the structure image are the square net pattern composed of the obtuse zigzag lines and the rod-like figures of roughly 0.3 nm in width all located in the middle of the elemental squares. As each rod is orientated to be normal to those in the neighbouring squares, the two dimensional unit cell is assigned as the large square indicated in Fig. 2, showing a good agreement with that expected from the diffraction data. Note the high contrast dots at every knot of the net structure. Both ends of the individual rods are related to the square sides at their re-entrant tips.

The structure of this crystal had been analysed using the modified multislice calculation of the electron diffraction

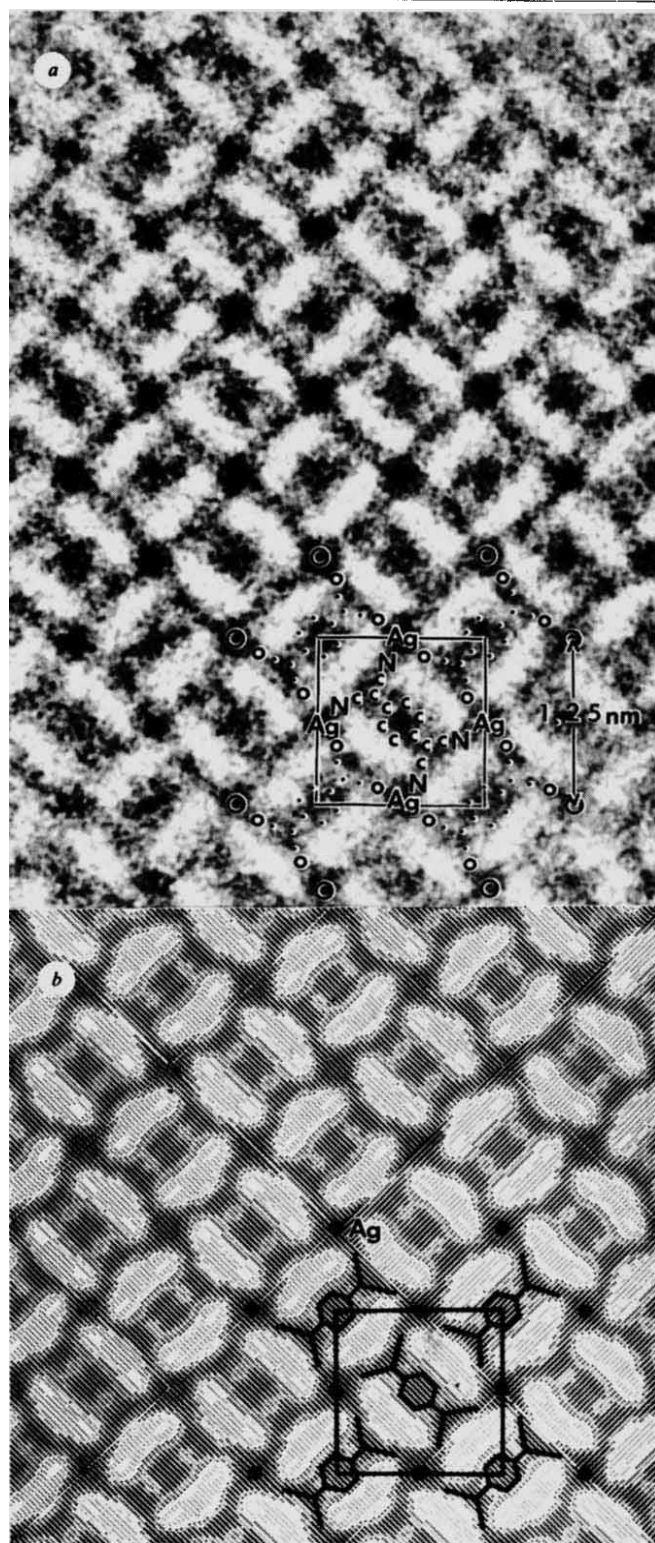


Fig. 2 a, Enlarged electron photomicrograph of Ag·TCNQ crystallite showing the images of Ag ions and TCNQ anions as indicated by the inserted molecular diagram. b, Computer-simulated images of the structure based on the projection as indicated by the schematic diagram. The imaging conditions of JEM-200CX are: the defocus spread, 15 nm, the illuminating angle, 0.1 mrad; and the Δf for the Scherzer focus, 55 nm.

intensities, although the details are not presented here. The molecular arrangement obtained as the preliminary result is shown in Fig. 2b which presents the theoretical images computer-simulated with the atomic parameters and the actual imaging conditions taken into account. Comparison of these two images clearly shows that the contrast distribution in the real images can be interpreted in terms of the molecular and ionic arrangements as indicated by Fig. 2a. The re-entrant side of each square corresponds to the chain of N-C-C-C-N, and the oblong rod can be regarded as the benzole ring, although they are all the columnar projections of 7–10 identical species. On the basis of this remarkable coincidence, the dark dot which is visible at every knot of the net structure can be regarded as the image of Ag ions superimposed in the direction of the column axis.

As far as the projection along a proper axis is concerned, this type of molecular and metal ionic arrangements show remarkable similarities to those of K, Na and Rb salts of TCNQ determined by Richard *et al.*⁵, Konno and Saito⁶, and Hoekstra *et al.*⁷, respectively. In particular, the K salt seems to show the closest resemblance as its projection along the *a*-axis of a monoclinic unit cell almost produces a tetragonal symmetry in the molecular and ionic arrangements. The TCNQ complexes of

other metals such as Al, Au, Cs, Cu, Li and Ni were found by SAD to assume structures which are all isomorphic to the present Ag salt when prepared by the solid state topotaxy. In fact, Hoekstra *et al.*⁷ has found by X-ray diffraction that the Li salt has a tetragonal unit cell although no detailed structure analysis has been performed.

The critical radiation dose ($\sim 0.3 \text{ C cm}^{-2}$) of the present complex is measured by applying 100-kV electrons to the fading time method. This dose is less than those for some aromatic compounds which are known to be rather radiation-resistant. However, the atomic resolution is attainable only if the minimum exposure technique is applied to those organic crystals which are radiation-resistant to this extent.

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Inelastic electron tunnelling spectroscopic study of lubrication

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Inelastic electron tunnelling spectroscopy reveals the vibrational spectrum of organic molecules on the oxide of a metal-oxide-metal junction^{1–3}. A vibrational mode of frequency ν appears in the tunnelling spectrum as a peak in the second derivative of voltage with respect to current, d^2V/dI^2 , at a voltage $V = h\nu/e$ (where h is the Planck's constant, and e is the electron charge). Both IR and Raman active modes can be observed. Tunnelling spectroscopy^{4,5} has been used as an effective surface probe in the studies of adhesion^{6–8}, biochemistry^{9,10}, water pollution¹¹, damage due to electron beam irradiation¹², or UV irradiation¹³, surface chemistry and catalysis^{14–18}. We report here a new application of tunnelling spectroscopy—to lubrication—and present spectra of 1,2 hexadecanediol, hexadecanol and hexadecanoic acid and relate the results to their properties as boundary lubricants for aluminium. Because diols give the best results in both friction measurements and tunnelling spectra, we studied the adsorption of the smallest diol, ethylene glycol, and found that both OH groups react with the alumina surface.

As the experimental details are described elsewhere², we only give an outline here. We evaporated an aluminium strip 700-Å thick onto a clean 1×3 inch glass slide and oxidized the aluminium strip by venting the chamber to pure O₂. When a thicker alumina layer was needed to produce a junction of the desired resistance we thermally oxidized the strip in air at 200 °C. We doped the oxidized aluminum strip either with a liquid solution of the molecules of interest or a vapour. Before vapour doping the alumina was cleaned in an argon glow discharge¹⁹. We completed the junction with a 3,500 Å thick evaporated lead cross strip.

We checked the electrical resistance of each junction with a low-power ohmmeter. Doped junctions with resistances in the range 100–1,000 Ω were acceptable. For the spectra to be taken the slide was mounted on the sample holder with small screw clamps which made the electrical connections to the evaporated electrodes. The junction assembly was then immersed into liquid helium at 4.2 K and tested for the proper current-voltage tunnelling characteristics to ensure that all the current flow was

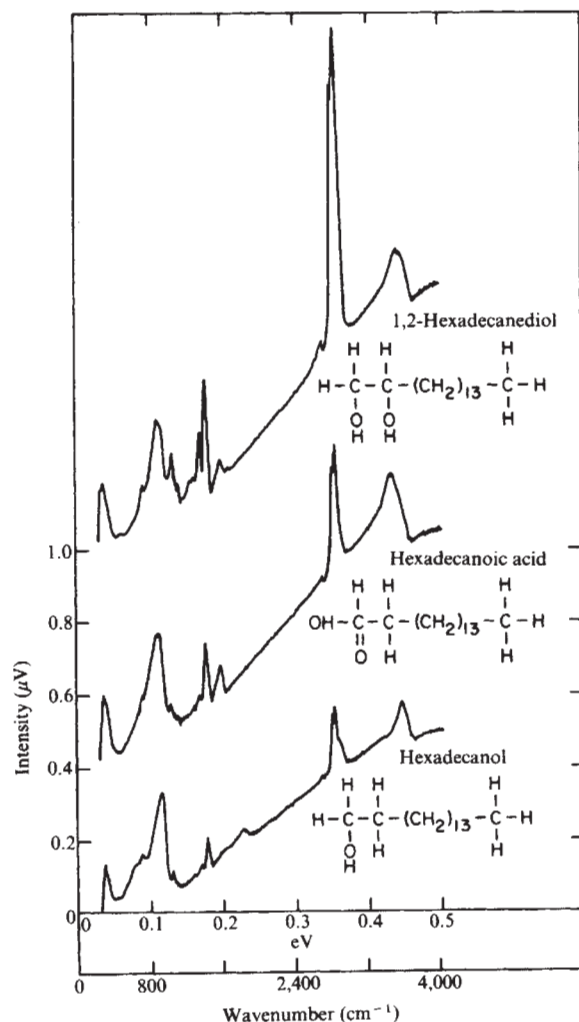


Fig. 1 Tunnelling spectra of C₁₆ molecules with different attachment groups. The compounds are doped at the same concentration, 0.05%, from an emulsion of 90% D₂O and 10% hexane.

due to tunnelling. Second derivative spectra were then obtained by detecting the second harmonic voltage due to a small modulation current with a lock-in amplifier as a function of voltage.

Figure 1 shows the inelastic electron tunnelling spectrum of hexadecanol, hexadecanoic acid and 1,2-hexadecanediol. The compounds were doped from an emulsion of 90% D₂O and 10% hexane at a concentration of 0.05%. Among the three spectra 1,2-hexadecanediol has the most intense peaks. It also gave the most reproducible results. The second harmonic intensity, plotted on the y axis, is an intensive quantity independent of junction area and resistance²⁰. The peak highs are a measure of surface coverage²¹. The acid was the most difficult to work with. It often gave shorted junctions indicating damage to the junction's oxide layer. The alcohol adsorbed least, as indicated by the relatively small peaks. From these observations, we guess that the diol would be the best lubricant: it had strong, reproducible adsorption without damaging the oxide.

Hotten's measurements of the load-carrying and wear-reducing properties of emulsions containing long-chain alcohols and diols show that the failure load is higher and aluminium

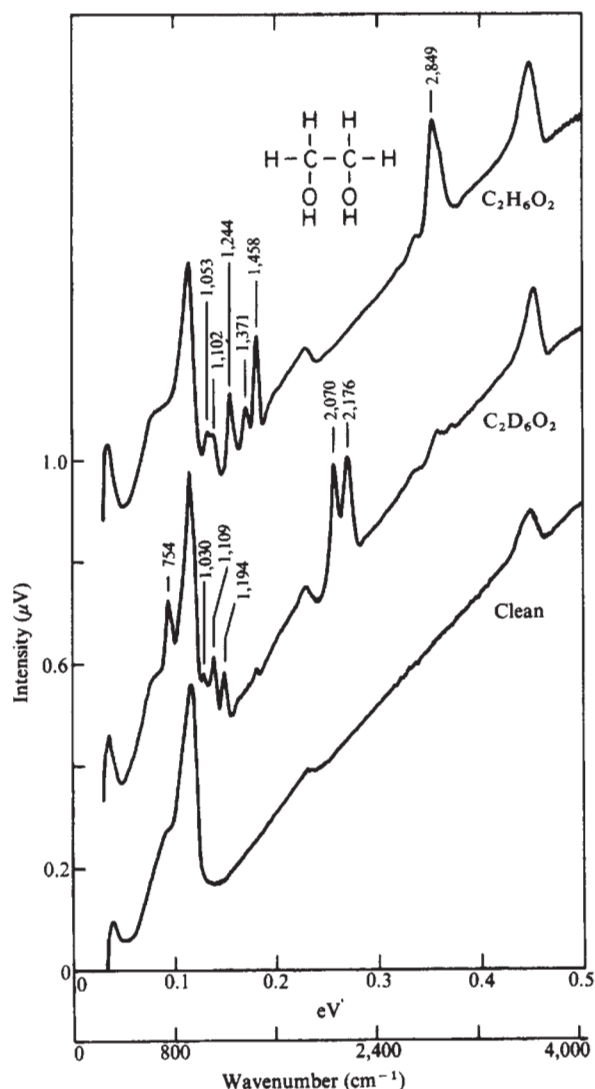


Fig. 2 Tunnelling spectra of ethylene glycol, deuterated ethylene glycol and a clean junction. The ethylene glycol and the deuterated ethylene glycol were doped at 1×10^{-3} torr and 3×10^{-3} torr for 1 min and 4 min respectively. The OH or OD vibrations of the diols are not present in the spectra implying that the hydroxyl groups lose their protons and attach to the oxidized aluminium surface through their oxygens.

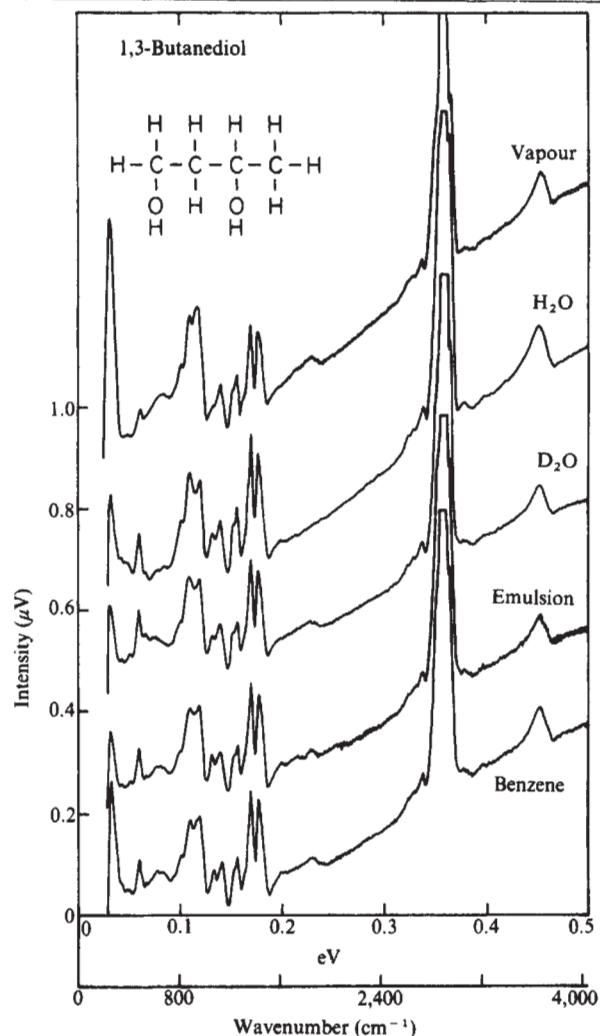


Fig. 3 Tunnelling spectra of 1,3-butanediol. The same surface species results from vapour doping or liquid doping from different solutions. The vapour doping was at 1×10^{-3} torr for 100s. The liquid doping concentrations were 25%, 50%, 12% and 20% by volume for the H₂O, the D₂O, the emulsion of 80% D₂O and 8% hexane, and the benzene respectively.

wear is lower (by a factor of ~ 3) for 1,2 and 1,3 diols compared with the corresponding alcohols²². Although there have been experiments to determine the lubrication properties of long chain acids on aluminium²³, no direct comparison is possible with the diols.

The emulsion that was used was meant to model the emulsions typically used in the lubrication of aluminium during rolling and cutting operations. An important difference is that D₂O was substituted for H₂O. This D₂O should have exchanged the two protons on the hydroxyl groups of the diol, the one on the carboxylate group of the acid; and the one on the hydroxyl group of the alcohol. Note, however, that there are no OD bands in the spectra. (They would be expected between 2,400 and 2,700 cm⁻¹.) This indicates that all these exchangeable protons are lost during bonding of the compounds. This was anticipated in the case of the alcohol and the acid. Greenler²⁴, Evans and Weinberg¹⁵, and Evans *et al.*¹⁶ showed that alcohols adsorb as alc oxides on alumina, losing the proton of their hydroxyl group. Klein *et al.*²⁵, Hall and Hansma²⁶, Brown *et al.*²⁷, Lewis *et al.*²⁸ and others showed that acids adsorb as carboxylate ions on alumina, losing the proton of their carboxylate group. Figures 1-3 show that diols lose protons from both hydroxyl groups.

Figure 2 shows the spectra of ethylene glycol, totally deuterated ethylene glycol and a clean junction. Ethylene glycol is the smallest molecule that has the same attachment group as 1,2-hexadecanediol. There are no peaks in the tunnelling spectra where the O-H and O-D vibrations for ethylene glycol should occur^{29,30} which shows again that the molecule loses the protons from both hydroxyl groups during bonding to the aluminium oxide surface. Unfortunately there are no IR or Raman spectra available for metal salts of ethylene glycol with which we can make a direct comparison of our spectra. The most we can say is that there is a good comparison between our observed peak positions and those of model compounds such as *p*-dioxane³¹ and glycol nitrate³² which contain the X-O-CH₂-CH₂-O-X structure.

Does the vapour doping technique that is useful for these small molecules give the same surface species as would be deposited from a lubricant solution? Figure 3 shows the tunnelling spectra of 1,3-butanediol vapour doped and liquid doped from various solvents. Every peak in the vapour-doped spectrum is present in the other four spectra which shows that the surface species formed is independent of the doping technique and solvent used. The similarity of spectra doped from both aqueous solutions to the others also suggests that the water is not chemically modifying the oxide layer: not converting the oxide layer to a hydroxide. Note that deuteration of the OH groups by the use of D₂O as a solvent gives the same spectrum as with H₂O. This is consistent with the results for ethylene glycol and 1,2-hexadecanediol.

Inelastic electron tunnelling spectroscopy can reveal the surface adsorbed species from model lubricant solutions. Long chain 1,2- and 1,3-diols which are effective boundary lubricants chemisorb to the aluminium oxide surface more readily than the corresponding alcohols or acids and they are less destructive to the oxide layer than acids. In diols both hydroxyl groups react with the alumina surface. The same surface species is formed from both vapour doping and liquid doping from polar and non-polar solvents and emulsions.

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Ferromagnetic coupling to muscle receptors as a basis for geomagnetic field sensitivity in animals

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Over the past decade several investigators have provided convincing evidence that the orientation of pigeons and other birds during homing and migrational activities is significantly affected by Earth-strength (≤ 0.5 G) magnetic fields¹⁻⁸. The presumed mediator of such effects would be a highly sensitive magnetoreceptor which the birds would normally use to extract navigational information from the geomagnetic field. The recently reported measurement of magnetic remanence in honeybees⁹ and in homing pigeons¹⁰ has stimulated interest in the possibility that the magnetically sensitive structure may be constructed from permanently magnetic material. Here we report the detection of permanently magnetic material in the neck musculature of pigeons (*Columba livia*) and migratory white-crowned sparrows (*Zonotrichia leucophrys*). We propose that a magnetic field detector might involve the coupling of magnetic particles to a sensitive muscle receptor such as a spindle. A detection mechanism of this kind could account for the difficulties encountered in conditioning immobile homing pigeons to magnetic field changes^{11,12} and for the puzzling requirement of movement in other behavioural experiments involving pigeons and magnetic fields^{13,14}.

As behavioural experiments suggest that the magnetically sensitive structure in the homing pigeon might be located in the head or neck^{1,2}, we concentrated on this region. We tested for the presence of permanently magnetic material by measuring specimens in a SQUID (Superconducting Quantum Interference Device) magnetometer, an instrument used extensively by geologists to measure palaeomagnetic remanence in rocks¹⁵ and also used in earlier studies of honeybees⁹ and pigeons¹⁰. Birds were freshly perfused with phosphate-buffered formaldehyde (4%, pH 7.4), as the acid pH of unbuffered fixative might attack magnetic iron oxides. All dissections were conducted with clean glass microtome knives to prevent possible contamination from metallic instruments. To maximize the signal from any permanently magnetic material present, specimens were exposed to a 1,000-G field from a small cobalt-samarium magnet immediately before being measured in the magnetometer. This exposure has the effect of increasing the alignment of any permanently magnetic moments; the resulting measurement is termed inducible magnetic remanence.

Eleven homing pigeons from our Caltech loft (eight of local stock; three of an Oregon stock specifically chosen for their ability to orientate in overcast conditions; aged from 12 days to 12 years) and eight local feral pigeons were tested using the SQUID magnetometer. Inducible remanence of magnitude 1.0×10^{-6} to 1.5×10^{-5} e.m.u. was found in the head and neck area of all 19 pigeons tested (Table 1). Within the head, reproducible remanence was found to be associated with the skull. This remanence was diffuse, being spread rather uniformly throughout the entire skull; we could find no visible magnetic structures in the skull. This is in contrast to the results of Walcott *et al.*¹⁰ who found permanently magnetic material in the pigeon head to be concentrated in a small, unilateral, innervated piece of tissue between the dura and the skull. We also found reproducible inducible magnetic remanence in the neck musculature of the pigeon, especially in the complexus muscle and associated fascia (Table 1).

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Seven migratory white-crowned sparrows (all adults at least one season old) were also tested for inducible magnetic remanence in the head and neck (Table 1).

In addition to pigeons and white-crowned sparrows, frozen specimens of approximately 40 other species of birds were tested for inducible magnetic remanence in the head and neck. Widely varying results from $<10^{-6}$ to $>10^{-5}$ e.m.u. were found; however, all cases of appreciable remanence were associated with migratory birds (for example, tree swallow [*Iridoprocne bicolor*], 5×10^{-6} e.m.u.; western grebe [*Aechmophorus occidentalis*], 9×10^{-6} e.m.u.; pintail duck [*Anas acuta*], 6×10^{-6} e.m.u.).

We were able to visualize magnetic material imbedded in the neck musculature of three homing pigeons (aged 3, 11 and 12 years) and two white-crowned sparrows. This material had the form of diffuse patches of black magnetic material in fascia or muscle (Fig. 1) or particles of black magnetic material embedded in muscle fibres. The black magnetic material was sometimes found together with transparent orange crystals possibly similar to those associated with magnetic material in chiton teeth^{17,18} and also reported by Walcott *et al.*¹⁰ in pigeons. Electron probe X-ray analysis (MAC-5-SA3 electron microprobe) of magnetic material from the neck musculature of pigeons and white-crowned sparrows indicated that iron was the major elemental component. This, together with the black colour and appreciable permanent magnetism, suggests that the material is probably composed largely of magnetite (Fe_3O_4), in agreement with the conclusions of previous reports of magnetic remanence in honeybees⁹ and in pigeons¹⁰. Remanence of 1.0×10^{-6} e.m.u. could be produced by approximately 6×10^6 $0.07\text{-}\mu\text{m}$ cubic single domain particles of magnetite¹⁶ (amounting to 10^{-8}g), provided their magnetic moments were all aligned. If the magnetic domains interact, adjacent moments would tend to cancel and a larger amount of magnetite would be required to produce the same remanence.

Geomagnetic field sensitivity in animals may arise from the coupling of small permanent magnets to sensory structures which originally evolved to serve other functions. Animal skeletal muscle is richly innervated with sensory fibres which supply receptors sensitive to pressure and stretch. One such receptor type, the muscle spindle, is acutely sensitive to stretch and has a major role in the regulation and perception of muscle movement, both at the spinal and supraspinal levels¹⁹⁻²¹. We propose that permanently magnetic grains, coupled to such a muscle receptor so that the torque exerted on the grain ensemble in the geomagnetic field is sufficient to stimulate the muscle receptor, might form an effective basis for magnetic field sensitivity. This proposal is, of course, motivated by our finding of significant reproducible magnetic remanence and visualizable iron-rich magnetic material in the neck musculature, although we think it unlikely that the large visualizable deposits in the older birds are more than epiphenomenal.

One important implication of a muscle spindle-based magnetic field sensor is that natural motion of the muscle may be requisite for the perception of magnetic stimuli. This suggestion is based on the findings of several experiments with humans which indicate that perception of muscle spindle discharge is

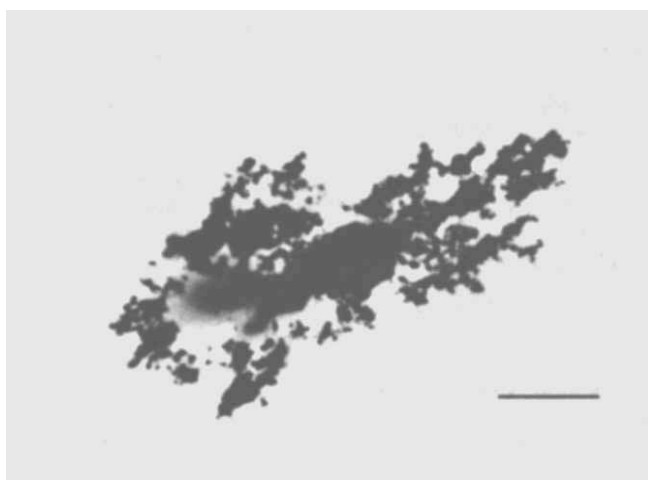


Fig. 1 Highly magnetic black particles (induced remanence of approximately 15×10^{-6} e.m.u.) found between the surface of the complexus muscle and the superficial fascia of a 3-yr-old homing pigeon. The particles are imbedded in a sheet of fascia. Unstained whole mount. Scale bar, $25\text{ }\mu\text{m}$.

indirect, and seems to be possible only with reference to movements initiated by the subject²²⁻²⁴. This might account for one slightly puzzling aspect of geomagnetic field sensitivity in homing pigeons; namely, the failure of several investigators to condition a cardiac response to magnetic stimuli in the laboratory^{11,12}. In these experiments the pigeon is held immobile in the centre of coils with which the ambient magnetic field can be altered. Also intriguing are the results of laboratory experiments by Bookman^{13,14}. He found that homing pigeons could be trained to discriminate between the presence and absence of an Earth-strength (0.5 G) magnetic field and that successful discrimination was accompanied by fluttering (hovering, short flights) of the birds during the testing. If muscle activity of the sort normally executed by the bird during flight is required for the detection of magnetic stimuli, then the rather enigmatic results of Bookman's experiments would be readily explained.

It has not escaped our notice that our proposed mechanism may be relevant to the claim that positive dowsing responses in humans, indicated by the sudden flicking of a forked stick or the swinging of a pair of L-shaped rods, are correlated with relatively small changes in the local magnetic field²⁵⁻²⁸. As the dowsing instruments are held in such a way that the slightest movement of the wrists or forearms will be amplified into an appreciable movement of the stick or rods, a magnetically sensitive muscle receptor could conceivably give rise to muscular movement sufficient to produce the classic dowsing responses.

We thank Luis Baptista for providing the white-crowned sparrows, Eugene Cardiff for providing the frozen specimens and Joe Kirschvink and Maureen Steiner for introducing us to SQUID magnetometer methodology. This work was supported by a President's Venture Fund grant and Weizmann Fellowship to D. P. and USPHS grants to J. D. P.

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Table 1 Inducible magnetic remanence in the head and neck musculature of pigeons and white-crowned sparrows

	Head (e.m.u.)	Neck musculature (e.m.u.)
Pigeon	$1-7 \times 10^{-6}$ (19)	$1-15 \times 10^{-6}$ (19)
White-crowned sparrow	$2-4 \times 10^{-6}$ (2) 6×10^{-7} (5)	$1-8 \times 10^{-6}$ (7)

The numbers of birds which were found to have the various remanence values are shown in parentheses. For comparison with neck muscle specimens, comparably sized specimens of pectoral muscle and associated fascia from both pigeons and white-crowned sparrows had inducible magnetic remanences of less than 6×10^{-7} e.m.u. The noise level of the magnetometer was about 5×10^{-8} e.m.u.

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Evidence for fixed charge in the nexus

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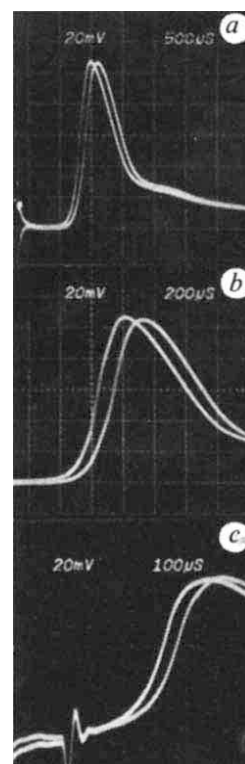
The nexus or gap junction has been characterized as a low-resistance junction as well as a highly permeable junctional membrane to many molecules¹⁻⁶. The transfer of electrical current from one cell interior to another, the aqueous solubility of dyes used to trace cell to cell communication and the fact that these molecules move across the nexus more rapidly than the plasma membrane⁴⁻¹⁰ have led to the hypothesis of an aqueous channel in the junction. Both Ca^{2+} (ref. 11) and H^+ (ref. 12) are thought to alter nexal membrane conductance, and a voltage-sensitive gate has been demonstrated within the junction¹³. Recently, Flagg-Newton *et al.*¹⁴ have concluded that mammalian junctions may contain fixed charge or be of smaller diameter than arthropod junctions. Here, we have investigated these alternatives by examining the permeability of nexuses of septa of the median giant axon of *Lumbricus terrestris*^{1,15} with various derivatives of fluorescein. Both carboxyfluorescein and aminofluorescein were found to have depressed permeabilities relative to their predicted permeabilities based on molecular size and weight (MW). Fluorescein diffusion was significantly suppressed in axons pre-injected with aminofluorescein but carboxyfluorescein had no such effect (Table 1). These data suggest the existence of fixed anionic charge within the nexal channel which may have affinity for amino groups.

Figure 1a and b illustrate the propagation of action potentials at the septa. The microelectrodes were 500 μm from the septum on either side; the position of the septum was delineated by a fluorescein injection. The action potential propagated across the septum at 8.0 m s^{-1} . Figure 1c shows the conduction of an action potential along the same axon in the absence of a septum. Here the conduction velocity was 25 m s^{-1} . The microelectrode separation was 1 mm. The velocity of the propagated action potential across the septum is reduced to 32% of the velocity measured along the axon. Note the slight loss in amplitude of the action potential after it has traversed the septum. Propagation of an action potential in either direction showed the same loss in amplitude. In Fig. 1c no loss in amplitude is observed when the action potential is conducted along the axon. The introduction of carboxyfluorescein, aminofluorescein or any of the halogen derivatives did not increase the time delay at the septum. Nexal membrane conductance was measured in the presence of carboxyfluorescein and aminofluorescein, and no statistically significant alteration in conductance was observed over controls⁴.

Figure 2 illustrates the nexal permeabilities of various molecules plotted as a function of their widest dimension. With increasing size, molecules have more difficulty traversing the

junction. However, in the case of carboxyfluorescein and aminofluorescein this relationship does not hold. One possible conclusion is that the extra carboxyl and amino groups are interacting electrostatically with fixed charge in the nexal pore, resulting in decreased junctional permeability for those molecules. The hydration radius of the two molecules would be expected to be greater than that of fluorescein by virtue of their greater polarity¹⁶ and this may in part explain their suppressed permeabilities. It must be remembered that all the probes to a greater or lesser degree are also hydrated. In contrast to carboxyfluorescein and aminofluorescein, tetrachlorofluorescein permeability on the basis of size (1.0 nm) and MW (471) would have a predicted permeability similar to those of dibromofluorescein or diiodofluorescein. Data given here show the permeability to be between those of dibromofluorescein and diiodofluorescein. The data in Fig. 2 also suggest a functional channel diameter of 1.2 nm.

Aminofluorescein binds to cytoplasmic proteins¹, and affinity for cytoplasmic proteins in part explains the suppressed permeability of aminofluorescein. It may also have an affinity for membrane proteins. However, carboxyfluorescein has little binding affinity for cytoplasmic proteins (Table 1). For



Aminofluorescein

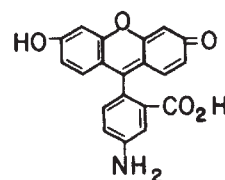


Fig. 1a An action potential recorded by two microelectrodes 1 mm apart with a septum between. The amplitude of the action potential is decreased slightly on the 'postsynaptic side' of the septum. The time delay across the septum was the same regardless of the direction of action potential propagation. Microelectrode resistance varied from 20 to 40 M Ω . Dye injections were done to confirm septum position. Dye was injected by applying hyperpolarizing current pulses for 15-30 min. b, As in a except 100 $\mu\text{s cm}^{-1}$. The conduction velocity in the region of the septum was 8 m s^{-1} . c, An action potential recorded between two microelectrodes 1 mm apart with no septum between. Note that the action potential shows no alteration in amplitude. The conduction velocity in the septum-free region was 25 m s^{-1} . Aminofluorescein contains an amine group in the γ position relative to the primary carboxyl group. Carboxyfluorescein substitutes a carboxyl group for the amine.

carboxyfluorescein, its extra electronegativity can be hypothesized to cause the molecule to experience increased repulsive forces in the region of the nexal channel, hindering its flux across the nexus. It can be speculated that aminofluorescein has a suppressed flux because the amine group is interacting with an anionic site in the junctional channel itself, but its size does not allow complete blocking of the channel.

Aminofluorescein or carboxyfluorescein was pre-injected into an axon using the method reported by us¹. Because both molecules diffuse across the septa slowly, within an hour only small or trace amounts of dye had traversed the septum into adjacent cells. Within that hour, fluorescein was injected (15 min) and post-injection photographs were made at 5-min intervals for 15 min after fluorescein injection. A photograph of the pre-injection of aminofluorescein or carboxyfluorescein was made immediately after its injection. The densitometric tracing of the pre-injection was subtracted from subsequent densitometric tracings made following fluorescein injection¹. Aminofluorescein significantly reduces fluorescein permeability whereas carboxyfluorescein has no such effect (Table 1). If there was significant diffusion of aminofluorescein or carboxyfluorescein into adjacent cells, it would result in an apparent elevation of the permeability, not a suppression of it.

No rectification is seen across the septum with propagated action potentials or with applied current pulses⁴. Likewise, no preferred diffusion or polarity was observed for any of the dyes. The nexal pores discriminate on the basis of charge and size but not with respect to the direction from which the molecule approaches and crosses the junction. If the nexal membrane is assumed to have a transnexal potential of 0, then the fixed charge could be placed near the cytoplasmic openings or anywhere along the interior of the channel and no rectification would be observed¹⁷.

The resistance for the earthworm septal membrane is $4 \times 10^4 \Omega$ (ref. 4). Electron microscopic data have revealed that the septa contain nexuses over 4–5% of their surfaces¹, with a total of 3×10^6 particles per septum. Assuming that all the nexal channels are in parallel and similar in size, the resistance of each channel is $1.2 \times 10^{11} \Omega$ or 8 pS. However, assuming the channel is 1.5 nm in diameter and filled with axoplasm having a resistivity of $200 \Omega \text{ cm}$ (r_a), the channel resistance (R_p) would be $2.3 \times 10^{10} \Omega$ or 43 pS using the equation

$$R_p = r_a L / A \quad (1)$$

where L and A symbolize length and cross-sectional area of the channel, respectively. The conductance of a single potassium

Table 1 The influence of aminofluorescein on nexal membrane permeability

Dye	Permeability (cm s^{-1})	Standard deviation (n)	Cytoplasmic binding
Fluorescein	5.4×10^{-5}	2.1×10^{-5} (10)*	
Aminofluorescein + fluorescein	2.2×10^{-5}	1.8×10^{-5} (11)*	
Carboxyfluorescein + fluorescein†	4.6×10^{-5}	1.3×10^{-5} (4)	
Aminofluorescein	6.0×10^{-7}	3.0×10^{-7} (8)	
Carboxyfluorescein	5.6×10^{-6}	3.0×10^{-6} (6)	6.8%
Tetrachlorofluorescein	2.1×10^{-5}	1.5×10^{-5} (4)	4.0%

Cytoplasmic binding was measured by the method given in ref. 1. The ratio of protein (cytosol) to dye was 10 to 1 assuming the average protein/MW was 10^4 – 10^5 . The control permeability value for fluorescein in the absence of other dyes was taken from ref. 1. Aminofluorescein was injected into an axon followed by a fluorescein injection. The aminofluorescein injection was accomplished by applying a hyperpolarizing pulse of 20 nA amplitude, 100 ms duration, every 150 ms for 20 min. The fluorescein injection followed immediately with the same conditions. The pH of the electrode solutions was 8.2. Permeability was calculated by the method reported by us¹. Aminofluorescein diffuses at such a slow rate that only trace amounts appear in the adjacent cell over an hour, allowing fluorescein to be injected (15 min) and photographs of subsequent fluorescein diffusion to be made at 5-min intervals for 15 min. Densitometric tracings were made of negatives and permeability calculated. Each photographic exposure was 40 s.

* Student's t -test comparison between fluorescein permeability and fluorescein permeability with a pre-injection of aminofluorescein. $P > 0.01$.

† Carboxyfluorescein was pre-injected using the same conditions as aminofluorescein followed by fluorescein injection. Fluorescein permeability and fluorescein permeability of carboxyfluorescein-injected axons were statistically insignificant.

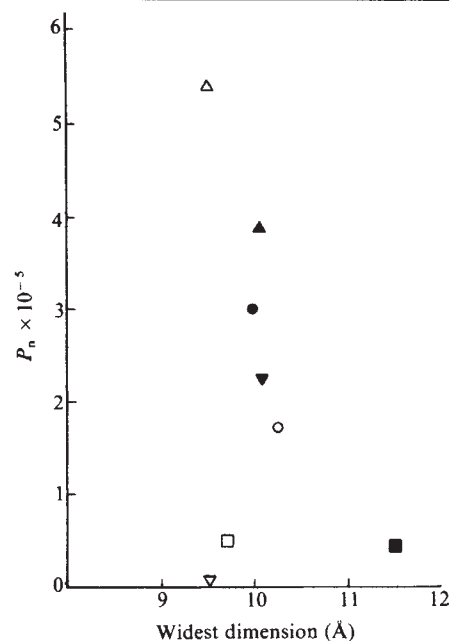


Fig. 2 Nexal membrane permeability plotted against the widest dimension of the molecules. Carboxyfluorescein ($\square$) and aminofluorescein (∇) are the only molecules that do not fit the general relationship of decreasing P_n with dimension or as MW increases for anionic molecules¹. The permeabilities of fluorescein (Δ), dichlorofluorescein ($\blacktriangle$), dibromofluorescein ($\bullet$), diiodofluorescein ($\circ$) and tetrabromofluorescein ($\blacksquare$) were taken from ref. 1. The permeabilities of carboxyfluorescein, aminofluorescein and tetrachlorofluorescein ($\blacktriangledown$) were computed using the same methods as in ref. 1. Carboxyfluorescein contains an extra negative charge at physiological pH whereas aminofluorescein exists as a zwitterion. Even with these additional groups the widest dimension of the derivatives is the same as fluorescein (9.5 Å), assuming no hydration radius. The carboxyl group of fluorescein has a $pK = 3$ –4 (ref. 20). Tetrabromofluorescein has a $pK = 5$ (ref. 21). Aminofluorescein would have a $pK = 3$ –4 while the amine group would be assumed to have a $pK = 10$. The conductance of the junction in the presence of the halogen derivatives fell within the range of junctional conductance with none of the fluorescent probes present. Permeabilities not listed in Table 1 are given in ref. 1.

channel was estimated by Cole¹⁸ from $1/f$ noise of hyperpolarized node membranes to be 0.5 pS. The sodium channel conductance has been given a similar value¹⁹. The diameter of the potassium channel is thought to be 0.3–0.4 nm. Using equation (1), where $r_a = 200 \Omega \text{ cm}$ and $L = 10.0 \text{ nm}$, the single channel conductance is 2.5 pS. The lower conductance value can be attributed to electrostatic forces acting over molecular distances in the potassium channel and nexal membrane channel. The data presented here therefore suggest the existence of an anionic site within the nexal membrane channel.

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Allergic encephalomyelitis induced in guinea pigs by a peptide from the NH₂-terminus of bovine P₂ protein

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Experimental allergic neuritis (EAN) is an inflammatory demyelinating disease of the peripheral nervous system (PNS) first described by Waksman and Adams^{1,2} in rabbit, guinea pig and mouse after injection of PNS antigen in Freund's complete adjuvant. In rabbit and mouse, the histological lesions were confined to the PNS but the guinea pig reacted curiously to PNS antigens by developing lesions in the central nervous system (CNS) as well as the PNS. Experimental allergic encephalomyelitis (EAE), the CNS counterpart of EAN, is known to be a response to CNS myelin basic protein. In early experiments^{3,4}, basic proteins from PNS myelin also produced a disease in guinea pigs characterised by CNS lesions. Characterisation of the proteins of PNS myelin demonstrated the presence of a basic protein (P₁) which was similar to the encephalitogenic basic protein of CNS myelin⁵. At first it seemed that the CNS lesions were being caused by this (P₁) protein. However, immunological studies from our laboratories suggested that when guinea pigs were sensitised to PNS myelin, the resulting lesions were a response to the small PNS myelin basic protein (P₂) and not to the P₁ protein⁶. In a preliminary report⁷, we indicated that a peptide fraction derived from the P₂ protein could produce a disease in guinea pigs characterised by CNS lesions. Sequence studies under way in our laboratory now enable us to report that this response is being caused by a tryptophan-containing 20-residue peptide (CN3) derived from the NH₂-terminal segment of the bovine P₂ protein.

Bovine P₂ protein prepared from spinal nerve roots⁸ has three methionine residues. Treatment of P₂ protein with CNBr results in four peptide chains, two of which remain covalently linked by an interchain disulphide bond. These chains consist of a peptide of 20 amino acids from the NH₂-terminus (CN3), a peptide of approximately 88 residues located internally (CN1), and the disulphide-linked dipeptide (CN2) representing the COOH-terminal 18 amino acids⁹. The sequence of the NH₂-terminal 65 residues which include CN3 has been reported¹⁰.

In early experiments¹¹, P₂ protein (55 mg) was digested with CNBr (in 3.0 ml 70% formic acid) and the resulting peptides were separated by chromatography on a column (1.5 × 69 cm) of

Encephalitogenic determinant

Residues 114–122 of bovine CNS MBP

PHE-SER-TRP-GLY-ALA-GLU-GLY-GLN-LYS
114 122

Peptide CN3

Residues 1–20 of PNS P₂ protein

N-AC-SER-ASN-LYS-PHE-LEU-GLY-THR-TRP-LYS-LEU-
1
VAL-SER-SER-GLN-ASN-PHE-ASP-GLU-TYR-MET
20

Fig. 1 Sequences of the encephalitogenic determinant of bovine CNS myelin basic protein and peptide CN3 of bovine PNS myelin P₂ basic protein.

Sephadex G-50. The column was equilibrated and eluted with 0.1 M acetic acid at 35 ml h⁻¹; 3.0-ml fractions were collected. Only two main peptide-containing peaks were isolated. The peak appearing in the void volume (fractions 10–35) was designated fraction I and contained only the large internal peptide CN1. The second peak (fractions 38–43) was designated fraction II and was later shown to be a mixture of peptides CN2 and CN3. Each of these fractions was tested for its ability to produce disease in guinea pigs (Table 1). Of the two, fraction II clearly contained a disease-inducing site for guinea pigs, and produced a response characterised by strong clinical symptoms (at 75 µg dose) and histological lesions in the CNS characterised by unusually large numbers of plasma cells in the infiltrates as compared with either EAE or EAN.

In later experiments⁹, the peptides resulting from CNBr digestion of the P₂ protein were separated by chromatography on G-25, which allowed isolation of CN2 and CN3. To establish which of the two contained the disease-inducing site, both were tested at two doses in guinea pigs. The data in Table 1 suggest that the determinant for disease induction in the guinea pig resides in the NH₂-terminal 20 amino acids of P₂ protein that comprises peptide CN3. The response in one of the three guinea pigs sensitised with CN3 also resulted in histological lesions in the CNS characterised by infiltrates containing unusually high numbers of plasma cells.

Because P₁ is encephalitogenic¹², the question of contamination of peptides derived from P₂ with the encephalitogenic determinant of P₁ must be considered. Our P₂ preparation is

Table 1 Disease-inducing properties in guinea pigs of CNBr peptides derived from the basic P₂ protein

Antigen	Dose (µg)	No. of animals tested	No. with clinical signs (day of onset)	No. with perivascular infiltration (severity) CNS	Histology PNS	No. with demyelination CNS	No. with demyelination PNS
Fraction I (CN1)	50	3	0	2 (+/-)	0	0	0
Fraction II	75	4	4 (12, 12, 14, 19)	4 (++)*	0	4	0
	50	4	1 (15)	4 (+/- to +)*	0	2	0
CN2	75	2	0	0	0	0	0
	25	3	0	0	0	0	0
CN3	75	3	1 (15)	3 (+/- to +)	0	1	0
	25	3	0	2 (+/- to +)	0†	2	0

Each animal was injected into each hind footpad with 0.1 ml (0.2 ml per animal) of an emulsion containing equal volumes of antigen dissolved in saline and Freund's complete adjuvant (Difco, H37 ra). Clinical signs and histology were evaluated as described previously¹¹. Perivascular infiltration was graded in severity according to the system of Alvord and Kies¹⁵.

* Plasma cells were unusually prominent in the mixed cellular infiltrates.

† A small focus of perivascular lymphocytes was observed in the nerve root of one animal.

assessed to be free of contamination with the encephalitogenic P₁ protein by its failure to produce allergic encephalomyelitis when injected into rabbits, even at 5 mg doses⁸. It is even more unlikely that CN3 is contaminated with the encephalitogenic determinant from P₁ because, after cyanogen bromide cleavage of P₁, this determinant would remain in a large (146 residue) peptide which would easily separate from the small (20 residue) CN3 during subsequent purification by column chromatography and paper electrophoresis. There is no evidence that CN3 contains additional peptides even after tryptic digestion⁹.

CN3 apparently contains the antigenic determinant in bovine P₂ responsible for producing a neurological disease in guinea pigs characterised by perivascular infiltration and demyelination in the CNS. The encephalitogenic determinant for EAE in the guinea pig has been shown¹³ to be a peptide (residues 114–122) of the bovine CNS myelin basic protein containing that molecule's only tryptophan residue (see Fig. 1). Tryptophan is essen-

tial for disease induction in conjunction with a Gln-Lys (or Gln-Arg sequence)¹⁴. By way of comparison, peptide CN3 (residues 1–20 of the bovine PNS myelin basic protein) also contains a single tryptophan, an Asn-Lys sequence and produces an EAE-like disease in guinea pigs. One might speculate that the chemical requirements of a determinant for EAE are not exact and that the presence of tryptophan, asparagine and lysine in CN3 may be sufficient for its disease induction. One might further speculate that the reason for the required large dose of this peptide and resultant milder disease (compared with the encephalitogenic peptide of CNS myelin basic protein) is that the relative locations of tryptophan and lysine residues are different.

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Structure of slow-reacting substance of anaphylaxis from guinea-pig lung

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Slow-reacting substance of anaphylaxis (SRS-A) is a primary mediator of immediate-type hypersensitivity reactions, probably having a major bronchoconstrictor role in asthma. Although it was discovered some 40 years ago¹ the structure has remained unknown. Major advances in purification technology^{2,3} have resulted in the preparation of chemically pure material, and the demonstration that SRS-A has a characteristic UV spectrum² has enabled a correlation to be made between biological activity and a structural moiety in the molecule. Previous studies have indicated that SRS-A from guinea pig lung is derived from arachidonic acid⁴; data, based on the chemical and enzymatic destruction of biological activity, have indicated the presence of α -amino, carboxyl and thioether functions³. We now report the first spectroscopic (mass spectrometric) and analytical protein chemical data on intact SRS-A which allows us to define unequivocally the complete covalent structure of this immunologically generated material as the novel peptidolipid 5-hydroxy-6-cysteinyl glycyl-7, 9, 11, 14-eicosatetraenoic acid. The structure determined is identical to that which we have assigned to the major non-immunologically generated slow-reacting substance (SRS) obtained from rat basophil leukaemia (RBL-1) cells⁵, but different from that of a recently synthesized chemical with SRS-like activity⁶ whose structure was modified from an earlier, incorrect structure of the natural product⁷.

SRS-A was prepared from immunologically challenged lungs of guinea pigs sensitized to ovalbumin⁸ and purified to homogeneity by our previously published methods^{2,3} (Fig. 1); the difficulties associated with the purification of vanishingly small amounts of SRS-A were overcome by use of the procedures shown in Fig. 1, including the introduction of reverse-phase HPLC^{2,8}, with its attendant high resolution separative capabil-

ity. It was shown that the essentially homogeneous material prepared had a characteristic UV spectrum² (Fig. 2) ($\lambda_{\text{max}}^{\text{MeOH}}$ 280 nm) and this discovery has been very useful in locating SRSs^{8,9} and providing a cross-correlation between biological activity on the guinea pig ileum and a chemical entity, the conjugated triene attributed to this chromophore⁸.

In the final purification (Fig. 1) the active SRS-A fraction from HPLC-1 separated³ into four UV-absorbing compounds (Fig. 2); compound I (λ_{max} 280 nm) fulfills all the criteria for SRS-A⁸; compound II has a similar UV spectrum, but is shifted hypsochromically by 2–3 nm (λ_{max} 278 nm). This material has apparently reduced biological activity, probably corresponding to a *cis-trans* isomerization of one of the double bonds in SRS-A; compound III (λ_{max} 270 nm) does not contract the guinea pig ileum and we have previously shown this to be 5, 12 dihydroxy-6, 8, 10, 14 eicosatetraenoic acid³, compound IV is probably related to compound III by a similar isomerization to that observed for compounds I and II.

The precursor role of arachidonic acid has been indicated by data on the potentiation of SRS-A from guinea pig chopped lung⁴. Destruction of biological activity by soya bean lipoxigenase (ω 6 specificity) has shown the presence of a 1–4 *cis* pentadiene unit in the SRS-A molecule¹⁰.

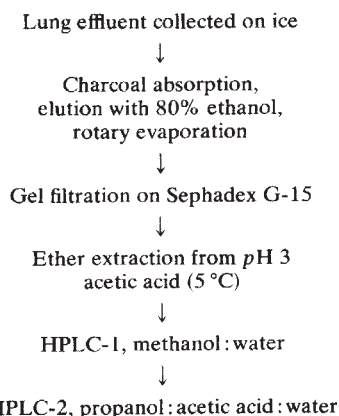


Fig. 1 The extraction and purification of SRS-A from guinea pig lung^{3,8}. At all stages of purification SRS-A was monitored by bioassay on guinea pig ileum (in the presence of mepyramine and hyoscine).

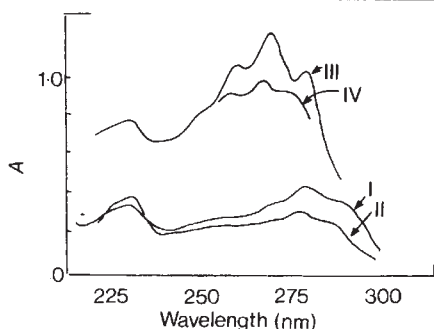


Fig. 2 The full UV spectra of compounds I-IV isolated by purification of the active fraction from immunologically challenged guinea pig lung.

Chemical inactivation data studied by monitoring the loss of biological activity after brief exposure of SRS-A to various reagents indicates the presence of certain functional groups³— α -amino (short acetylation), thioether (cyanogen bromide) and carboxyl (methylation). The presence of the α -amino group thus postulated in SRS-A, was used to provide a marker label in the mass spectrometric work (see below), thus enabling location of the relevant SRS-A signals and differentiation from those signals arising from background contribution.

Amino acid analysis of the peak tubes of biological activity showed the presence of the amino acids, glycine and cysteine. Tubes corresponding to compound III were devoid of amino acids above background level. To determine the sequence of the two amino acids, the active sample was dansylated, hydrolysed and the dansyl derivative examined by TLC¹¹. The results showed a fluorescent spot arising from a single N-terminus, moving more slowly than dansyl glycine in all dimensions; dansyl cysteine prepared as a standard in the presence of arachidonic acid gave a spot with identical mobility.

Derivatives for mass spectrometry were chosen to improve the volatility of SRS-A in a manner which would not affect the basic structural units, (1) by using reactions previously well studied in protein chemistry¹², and (2) by rejecting those reactions which caused changes in either UV absorbance or $\lambda_{\max}$ of the triene chromophore.

To monitor the variables of absorption phenomena, reagents and reaction conditions used in the final stages of the structure elucidation, a slow reacting substance (SRS), whose structure we have previously defined⁵, was prepared from rat basophil leukaemia cells (RBL-1).

The RBL-1 SRS had a similar elution position in the HPLC-2 step (Fig. 1), and identical pharmacological and physicochemical properties to SRS-A. The full UV spectrum of the SRS-A used for the mass spectrometric experiments was determined, (600 U, $A = 1.3$), and a deliberately smaller amount of RBL-1 SRS was taken for the control experiments, corresponding to 0.75 of the SRS-A UV absorbance. RBL-1 SRS was derivatized using acetic anhydride in the acetylation step, whereas the guinea pig lung SRS-A was acetylated using a 1:1 mixture of acetic anhydride and d_6 acetic anhydride to provide the mass spectrometric marker. The other derivative-forming steps were identical, being esterification in ethereal diazomethane (30 min) and trimethylsilylation in pyridine/bis(trimethylsilyl) trifluoroacetamide/trimethylchlorosilane (1:6:1 30 min).

Figure 3 shows the mass spectrum produced at 240 °C from guinea pig SRS-A (Fig. 3a) compared with that produced from the RBL-1 control (Fig. 3b) and with our previously reported spectrum of RBL-1 SRS, from which its structure was deduced⁵ (Fig. 3c). The spectra unequivocally show that SRS-A from guinea pig lung is identical to that of RBL-1 SRS in covalent structure, as the same fragment ions are observed in the same relative abundance ratios at an identical ion source temperature.

Figure 3 also illustrates the considerable difficulty of determining structure on the small quantity of SRS-A available from lung, for, in contrast to our previous work on RBL-1 SRS, at this

lower level the molecular ion was not observed for either SRS-A or the RBL-SRS control. The absence of the molecular ion does not in any way affect the interpretation, as the full structure is found by combining the information from the fragment ions formed. For example, in Fig. 3a, m/e 607 corresponds to $M^+ - OCH_3$ (containing both the peptide and lipid moieties), m/e 548 corresponds to $M^+ - TMSOH$ (showing the presence of a hydroxyl group), m/e 404/405 and m/e 314/315 ($-TMSOH$) define the lipid portion of the molecule and m/e 203 defines the substitution of the hydroxyl at C-5. Confirmatory experiments on the signals shown in Fig. 3, involving further isotopic labelling (for example, d_6 TMS), and high resolution mass measurements enable us to define rigorously the structure of SRS-A from guinea pig lung as the novel peptidolipid 5-hydroxy-6-cysteinylglycyl-7, 9, 11, 14-eicosatetraenoic acid (Fig. 4).

Using the same arguments as previously described in the structure elucidation of RBL-1 SRS⁵, we can position the double bonds in SRS-A and define the substitutions of the tetraene (C_{20} : 4-eicosatetraenoic acid) at C-5 (hydroxyl) and C-6 (thioether). Importantly, the mass spectrum also shows what is not present in the structure; for example, we can say that the SRS-A does not contain a glycine-amide which would be difficult to discount by chemical or enzymatic methods. Similarly, we can say that SRS-A does not contain a sulphate moiety, widely expected from the results of destruction of biological activity by aryl sulphatase, reported in the literature^{8,13}. It is of some interest that the structure we propose here for SRS-A derived from immunologically stimulated lung is identical to that which we have previously described for SRS released by non-immunological stimulation from malignant basophils. Furthermore, this structure could be biosynthetically derived by an intriguing combination of the lipoxygenase metabolism of arachidonic acid¹⁴ with the glutathione detoxification pathway¹⁵ involving nucleophilic attack of the

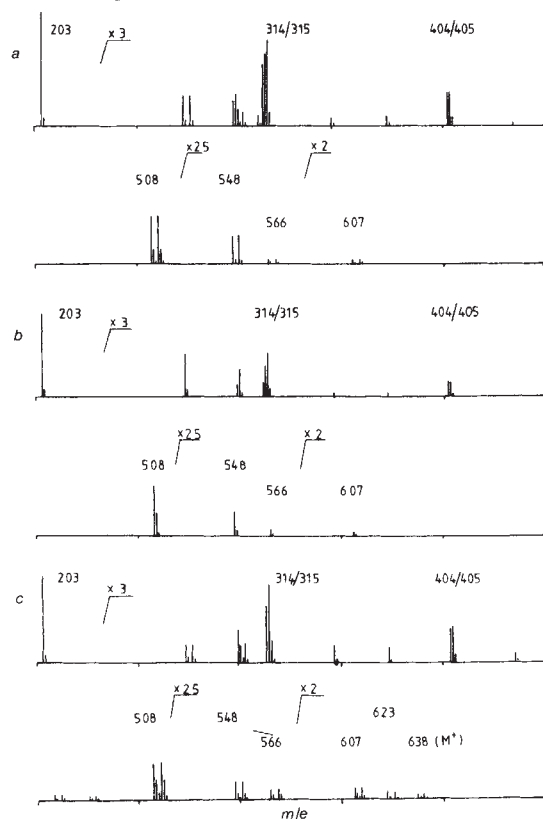


Fig. 3 (a), The electron impact mass spectrum of the trimethylsilyl (TMS) ether of the *N*-acetyl (CH_3CO/CD_3CO 1:1) methyl ester of SRS-A from guinea pig lung. Source temperature 240 °C. b, The mass spectrum of the same derivative (excluding isotopic labelling on the acetyl group) of the control sample of RBL-1 SRS. c, The mass spectrum of the same derivative of RBL-1 SRS⁵.

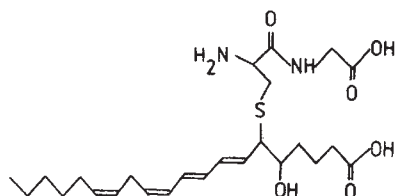


Fig. 4 The full covalent structure of SRS-A from guinea pig lung. Synthesis will be required to determine the full stereochemistry⁶. The detailed interpretation of the mass spectrum will be reported elsewhere.

cysteinyl sulphur on a 5,6-oxido-eicosatetraenoic acid. Interestingly, we find no evidence in either the natural SRS-A reported here or our previous SRS preparations⁵, for a glutathionyl structure such as that recently described for a synthetic substance possessing SRS-like pharmacological and chromatographic properties⁶. The cysteinyl glycine structure reported here (Fig. 4) is rigorously defined for both SRS-A and SRS⁵ from the mass spectrum (Fig. 3). We discount the possibility of having lost a major component of any putative glutathionyl SRS-(A) during purification, because (1) all fractions from the various column eluates were screened for biological activity, and (2) where partitioning took place (that is, at the charcoal absorption and ether extraction steps) no biological activity was found in the aqueous fractions. Despite yields of > 75% at these stages, this does not preclude either a minor component having adsorbed to charcoal (unlikely for a more polar substance) or enzymatic processing (either physiologically significant or otherwise) of compounds in the perfusate. In any event, the structure identified here for lung SRS-A is the major biologically active compound studied by pharmacologists and while the detailed enzymatic steps in the biosynthesis of SRS_s are under investigation, it is, however, possible that SRS_s of different cellular origins may have different structures or metabolism.

The first demonstration, given here, that a mass spectrum can be produced from undegraded SRS-A, taken together with our previous work on SRS⁵, forms the basis for future precise identifications of all these substances, using the readily formed derivatives suggested; the mass spectrum can also be used in quantitative studies.

This definition of the structure of guinea pig lung SRS-A and the likelihood that human SRS-A has the same structure⁸, open the way towards new and enlightened study into the pathology and therapy of asthma and other allergic diseases. It will also lead to exploration of the physiological roles of SRS-A, 5,12-dihydroxyeicosatetraenoic acid and the other UV absorbing lipoxygenase products.

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Chemical inducers of differentiation in Friend leukaemia cells inhibit lymphocyte mitogenesis

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Several phorbol esters, the potent tumour-promoting agents¹ isolated from croton oil, induce proliferation of human lymphocytes^{2,3} and enhance the mitogenic effect of lectins on bovine lymphocytes⁴. While studying the mitogenic properties of one of these agents, phorbol myristate acetate (PMA), we found that dimethyl sulphoxide (DMSO), frequently used as a solvent for PMA, markedly inhibits PMA-induced mitogenesis at DMSO concentrations that have little effect on phytohaemagglutinin (PHA)-induced responses. DMSO, as well as a variety of other organic compounds, induce erythroid differentiation in Friend leukaemia (FL) cells^{5–9}. Phorbol esters, on the other hand, are potent inhibitors of both spontaneous and induced cellular differentiation^{10–13}. We therefore investigated the relationship between the potency of compounds to induce erythroid differentiation in FL cells and their potency to inhibit lymphocyte proliferation induced by PMA and other mitogens. We report here that many of the compounds that induce erythroid differentiation in FL cells are similar to DMSO in selectively suppressing PMA-induced lymphocyte mitogenesis.

The effect of a variety of organic solvents on the proliferation of human lymphocytes stimulated with either PMA or PHA is shown in Table 1 and compared with their reported effects on the differentiation of FL cells. Compounds that induce erythroid differentiation in FL cells inhibit lymphocyte mitogenesis, whereas compounds inactive in inducing erythroid differentiation in FL cells do not inhibit lymphocyte mitogenesis. The concentrations of those compounds which suppress lymphocyte proliferation are in the same range as those reported to induce differentiation. Among the compounds outlined in Table 1, butyric acid, previously reported to inhibit lymphocyte activation¹⁴, is the most potent inducer of erythroid differentiation⁶ and is also the most active inhibitor of PMA-induced mitogenesis. However, compounds which are structurally related to butyric acid, but which do not induce erythroid differentiation,

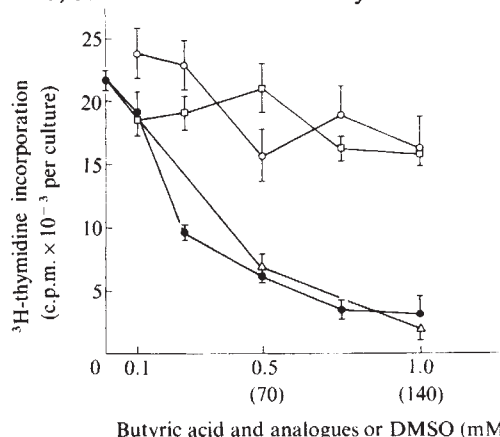


Fig. 1 Effect of butyric acid (●), DMSO (△), isobutyric acid (○) and α-aminobutyric acid (□) on the proliferative response of a human one-way mixed lymphocyte culture. The culture was done as previously described²⁶. This experiment is typical of three additional studies. Results are presented as the mean ± s.e.m. of quadruplicate cultures.

Table 1 Inhibition of lymphocyte mitogenesis by compounds which induce erythroid differentiation in FL cells

Compound tested	Concentration range (mM) resulting in 50% inhibition of ³ H-thymidine incorporation		Concentrations (mM) reported to induce maximum erythroid differentiation in FL cells
	PMA-induced responses	PHA-induced responses	
Polar organic solvents			
Dimethyl sulphoxide	60	>210	280(7)*, 280(9), 70–300(6)
Dimethylformamide	75	120	60(11), 150(7)
N,N-dimethylacetamide	5–10	25	20(9), 30(7)
Short-chain fatty acids			
Propionic acid	1–1.5	10	2(6)
n-Butyric acid	0.1–0.4	1.4	1–2(6), 1(9)
n-Valeric acid	1.6	>4.0	2(6)
Caproic acid	>10	>10	Inactive(6)
Butyric acid analogues			
Isobutyric acid	4–5	>10	30(24)
β-OH-butyric acid	>10	>10	Inactive(6)
γ-OH-butyric acid	>10	>10	
α-Amino-butyric acid	>10	>10	
β-Amino-butyric acid	>10	>10	
γ-Amino-butyric acid	>10	>10	Inactive(6)
Purine analogues			
Hypoxanthine	1–2	>5.0	3.7(15)
1-Methylhypoxanthine	0.5–1.0	3–5.0	3.3(15)
Xanthine	>5.0	>5.0	Inactive(15)
Allantoin	>5.0	>5.0	Inactive(15)

Human peripheral blood mononuclear cells were obtained from normal subjects as previously described²⁵. Final cell preparations (10^6 cells ml^{-1}) were suspended in RPMI-1640 medium containing 5% heat-inactivated fetal calf serum and supplemented with penicillin (100 U ml^{-1}) and streptomycin ($100 \mu\text{g ml}^{-1}$). Cells were distributed (0.2-ml aliquots) in flat-bottom microwells, the mitogens (PMA 10 ng ml^{-1} or PHA $2 \mu\text{g ml}^{-1}$) and compounds to be tested were added, and the cells were then incubated at 37°C in a 95% air, 5% CO_2 atmosphere for 72 h. ³H-thymidine incorporation ($2 \mu\text{Ci}$ per well) into DNA was determined during the final 52–72 h of incubation. The extent of proliferation as determined by ³H-thymidine incorporation in the absence of the test compounds was: PMA ($n = 76$) $91,169 \text{ c.p.m.}$ (s.e.m. = $\pm 2,312$), PHA ($n = 78$) $133,260 \text{ c.p.m.}$ (s.e.m. = $\pm 4,012$). *Numbers in parenthesis indicate reference from which data were obtained.

do not inhibit lymphocyte proliferation (Table 1). DMSO and butyric acid inhibit PMA-induced morphological transformation and ³H-leucine, ³H-uridine and ³H-thymidine incorporation.

The organic compounds do not inhibit lymphocyte mitogenesis by nonspecific effects on cell viability as directly assessed by the trypan blue exclusion assay. Moreover, following a 2-day incubation with 0.5–1.0 mM butyric acid or 70–140 mM DMSO, lymphocytes are capable of responding to mitogens when the organic solvents are removed by washing the cells. We also found that the organic solvents are most inhibitory when added early in blastogenesis and are much less effective when added 24 h after the mitogenic stimulus. Lymphocyte responses induced by other mitogens (concanavalin A, soybean agglutinin, peanut agglutinin and treatment with neuraminidase and galactose oxidase) have the same resistance to inhibition by DMSO and butyric acid as responses induced by PHA. Lymphocyte activation induced by allogeneic cells in one-way mixed lymphocyte cultures, on the other hand, are as sensitive to suppression by DMSO and butyric acid as are responses to PMA (Fig. 1).

Hypoxanthine and 1-methylhypoxanthine, both known to induce FL cell differentiation¹⁵, inhibit mitogenic responses to PMA and, to a lesser degree, responses to PHA. Xanthine and allantoin, on the other hand, are inactive in both assays (Table 1). Haemin, another known inducer of FL cells¹⁶, differs from the polar organic compounds in that it seems primarily to induce early events in differentiation¹⁷ and does not inhibit FL cell division¹⁸. We found that haemin alone induces modest increases in ³H-thymidine incorporation in human peripheral blood lymphocytes (Fig. 2). When cells are stimulated with PMA or PHA, haemin inhibits mitogenesis at approximately the same concentrations reported to induce FL cell differentiation (0.75–0.1 mM) (Fig. 2).

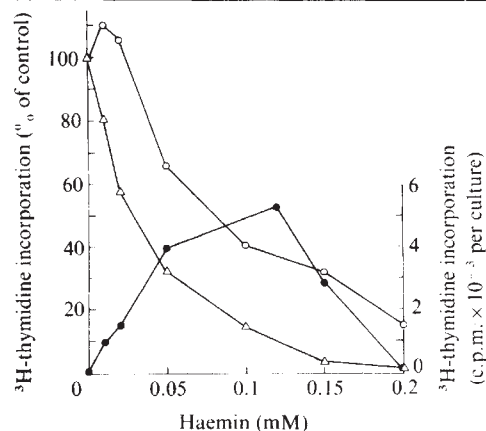


Fig. 2 Effect of haemin on the proliferative response of human peripheral blood lymphocytes (●), ³H-thymidine incorporation (c.p.m. $\times 10^{-3}$ per culture), and on PMA (Δ) and PHA (○) induced ³H-thymidine incorporation (% of control = % of ³H-thymidine incorporation in cultures stimulated with the mitogen in the absence of haemin). Results are presented as the mean of duplicate cultures. Three additional experiments gave similar results.

These studies indicate that there is a remarkable parallelism between the potency of compounds to induce erythroid differentiation in FL cells and their potency to inhibit lymphocyte mitogenesis. Inducers of erythroid differentiation have multiple effects on FL cells¹⁹ and it is unknown which of these effects are relevant to inhibition of mitogenesis. A prominent effect of the polar organic solvents is to decrease the fluidity of lipid membranes²⁰. The phorbol esters, on the other hand, increase the fluidity of cell membranes²¹, as do other mitogens²². It is possible, then, that the inducers of FL cell differentiation inhibit lymphocyte mitogenesis by interfering with the mitogenic signal at the membrane level. The selective inhibitory effect of the various inducers on lymphocyte responses to different stimuli might be due to different triggering signals induced by the different stimulatory agents, or to different subpopulations of lymphocytes responding to the different stimuli. An indication that PMA-responsive lymphocytes are distinct from those responding to PHA and concanavalin A was previously reported²³.

The chemical inducers of differentiation in FL cells constitute a new class of inhibitor of lymphocyte mitogenesis. Their potential use as immunosuppressive agents is now under investigation. A critical evaluation of the relationship between inhibition of lymphocyte mitogenesis and induction of differentiation should provide new insights into the mechanisms of these processes.

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Levels of 2,3-diphosphoglycerate in Friend leukaemic cells

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Most cells are thought to contain trace amounts of 2,3-diphosphoglycerate (DPG), as it acts as a cofactor in the interconversion of 2-phosphoglycerate and 3-phosphoglycerate by the glycolytic enzyme phosphoglyceromutase. DPG is synthesized from 1,3-diphosphoglycerate by the action of diphosphoglycerate mutase. Lowry *et al.*¹ reported levels of 29 $\mu\text{mol DPG per kg wet weight brain tissue}$ which is approximately 3 $\text{pmol per } 10^8$ cells, assuming that 1 g of brain tissue contains 10^8 cells. In contrast, erythroid cells contain 50–100 $\text{nmol DPG per } 10^8$ cells, depending on the species and the stage of development². This is of the order of a 1,000-fold more DPG compared with non-erythroid cells. In red cells DPG concentration modulates the binding of oxygen to haemoglobin^{3,4}. I show here that erythroid precursor cells also contain markedly raised levels of DPG.

The terminal stage of erythropoiesis is accompanied by the appearance of haem, globin, spectrin and carbonic anhydrase. There is evidence that the genes associated with these markers are expressed by virtue of a mitotic division⁵. Using Friend leukaemic cells (FLC) as a model of erythropoiesis⁶, this laboratory has attempted to obtain markers which characterize early cells in the erythroid lineage by studying the uninduced FLC. This cell seems to be arrested at about the proerythroblast stage of red cell differentiation⁷ unless it is induced to proceed further by a variety of polar compounds, generally dimethylsulphoxide (DMSO)⁸.

Anticipating that these erythroid precursor cells would develop mechanisms for accumulating iron before haemoglobin synthesis, the level of transferrin receptors in uninduced FLC was measured. These receptors bind transferrin specifically and facilitate its internalization together with two atoms of iron per molecule of transferrin. Our study⁹, and those of other laboratories^{10,11}, have shown that transferrin receptors are indeed present in uninduced FLC.

How iron is released from transferrin intracellularly is unclear. However, *in vitro* experiments using cell-free extracts have shown that DPG is able to mediate the release of iron from transferrin¹². Therefore, it is possible that this process takes place in the intact cell. If so, it would be expected that, in addition to red cells and induced FLC, erythroid precursor cells and uninduced FLC would display levels of DPG significantly higher than that found in non-erythroid cells. The level of DPG measured fluorimetrically¹ in uninduced FLC is 4.3 $\text{nmol per } 10^8$ cells (Table 1). This increases by about twofold after the cells are induced to differentiate by DMSO. In comparison, mouse red cells contain 81 $\text{nmol DPG per } 10^8$ cells. DPG in cultured embryonic mouse fibroblasts could not be detected using the standard assay, even after increasing the sensitivity of the assay 100-fold, that is, to the 10–50-pmol range. Similar results were obtained with cultured epithelial cells derived from embryonic rat hepatocyte cultures¹³ and cultured human lymphocytes of the IM-9 line¹⁴. It is concluded that these cells contain < 10 $\text{pmol DPG per } 10^8$ cells. This is less than reported for brain tissue¹, but in those experiments no precautions were taken to

Table 1 DPG levels in Friend leukaemic cells

Cell type	DPG (nmol per 10^8 cells)	% Hb cells
FLC (0)	4.3 $\pm$ 0.5	0.3
FLC (3)	5.2 $\pm$ 0.4	29
FLC (6)	6.1 $\pm$ 0.3	90
FLC (9)	8.7 $\pm$ 0.2	82
Mouse RBC	81.1 $\pm$ 3.2	100
Mouse fibroblast	ND	0
Rat epithelial cells	ND	0
Human lymphoid cells (IM-9)	ND	0

FLC were cultured as previously described⁹ in the presence of 1.86% DMSO for days indicated in parentheses. Duplicate estimations of DPG were performed on three cultures for each group and four samples for mouse RBC. Per cent haemoglobinized cells was determined by benzidine staining¹⁵. Between 5×10^7 and 10^8 cells were used for each sample. Cells were pelleted then washed twice with ice-cold balanced salts solution by centrifugation at 1,000g. The final pellet was sonicated in 100 μl 0.5 N HClO_4 , centrifuged at 4,000g for 10 min and the supernatant removed and neutralized with 100 μl 0.5 M NaOH. Aliquots of between 10 and 40 μl were taken for DPG determination by the enzyme-coupled fluorometric method of Lowry *et al.*¹. Each assay contained 5.2 nmol of NADH and the decrease in absorbance at 340 nm was measured in a Perkin Elmer fluorescence spectrophotometer model 240-A. DPG and NADH standards were incorporated in each set of determinations. Results are expressed as means $\pm$ s.e.m. DPG could not be detected (ND) in extracts of cultured embryonic mouse fibroblasts, rat epithelial cells of human lymphoid cells in spite of 100-fold increase in sensitivity over the standard assay. Therefore, these cells are estimated to contain less than 0.01 nmol DPG per 10^8 cells.

exclude possible contamination of samples with blood, whereas this is avoided in cultured cells. Reddy and Burns¹⁵ have attempted to measure DPG levels in myocytes, hepatocytes and adipocytes of the rat and conclude that levels were undetectable. They were also unable to demonstrate the presence of 2,3-diphosphoglycerate mutase, the enzyme responsible for DPG synthesis.

These results indicate that erythroid precursor cells, as judged by the levels found in uninduced FLC, have at least a 1,000-fold more DPG than non-erythroid cells; for example, rat epithelial cells, human lymphoid cells or mouse fibroblasts. The small percentage (0.3%) of haemoglobinized cells in the uninduced culture cannot account for the relatively high levels of DPG found in the control culture. This result differs from those of Kabat *et al.*¹⁶ and Scher *et al.*¹⁷, who were unable to detect DPG in uninduced or induced FLC using the method of Maeda *et al.*¹⁸. This may be explained by the greater than 100-fold increase in sensitivity of the method used in this study achieved by fluorometric measurement of NADH oxidation by enzyme-coupled assay in contrast to the spectrophotometric determination of inorganic phosphate liberated from DPG used in previous studies. After induction, when most of the FLC are haemoglobinized, there is only a small increase in DPG. The presence of DPG in uninduced non-haemoglobinized FLC suggests it is not one of the group of erythroid markers which appear at the terminal stage of erythropoiesis, as would be predicted if its only role in the red cell was to modulate the affinity of oxygen for haemoglobin. Its early appearance is consistent with the proposal that it may have a role in iron accumulation by the developing red cell and that it continues to accumulate during erythroid maturation.

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Saccharin may act as a tumour promoter by inhibiting metabolic cooperation between cells

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The possible role of saccharin in the carcinogenic process is, at present, still unclear. Carcinogenesis is a complex process involving, in many test systems, initiation and promotion phases¹. Current evidence favours the hypothesis that initiation is due to a mutagenic event, while promotion (at least the early portion) is the result of epigenetic changes². Although saccharin has been reported to be a weak mutagen in various *in vitro* test systems and a weak initiator in mouse skin³⁻⁷, there is increasing evidence from *in vitro*, as well as *in vivo*, studies that it might act as a tumour promoter⁸⁻¹³, rather than as a mutagen¹⁴⁻¹⁹. Recently, L.P.Y. *et al.*²⁰ and J.E.T. *et al.*²¹ developed an *in vitro* assay to detect tumour promoters, which has been independently reported by Murray and Fitzgerald²². The assay is based on the principle that phorbol ester-type tumour promoters block 'metabolic cooperation' or a type of cell-cell communication between cells. We report here a series of experiments demonstrating the elimination of metabolic cooperation in the hypoxanthine guanine phosphoribosyltransferase (HGPRT) system in Chinese hamster V79 cells, indicating that saccharin shares properties similar to those of other known promoters.

In an attempt to examine the potential biological effects of saccharin in intercellular communication, we seeded a small number of HG-PRT⁻ (6-thioguanine resistant) V79 cells in the presence of a large number of HG-PRT⁺ (6-thioguanine sensitive) cells sufficient to reduce the recovery of the resistant cells via the mechanism of metabolic cooperation²³. If the same experiment is conducted with the addition of 12-tetradecanoyl-13-phorbol-acetate (TPA) or a chemical with promoting properties, the reduction in the recovery of 6-thioguanine resistant cells is significantly prevented. Figure 1 depicts the result of adding various concentrations of saccharin (Sigma, Lot 65C-0129) to this metabolic-cooperation assay. With saccharin levels

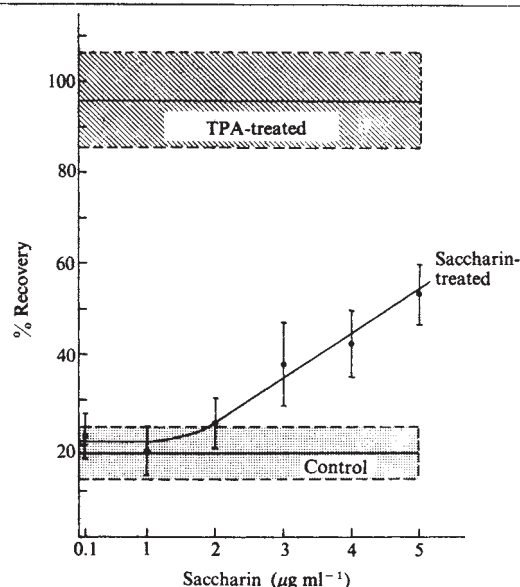


Fig. 1 Dose-response curve for the effect of saccharin on the inhibition of metabolic cooperation between 6-thioguanine sensitive and resistant Chinese hamster V79 cells. The shaded areas correspond to the mean recovery of 6-TG cells for the control and TPA-treated groups $\pm$ s.d. Wild-type s-TG⁺ cells and X-ray-induced 6-TG⁺ cells were grown in modified Eagle's medium supplemented with a 100% increase of non-essential amino acids, 1 mM sodium pyruvate and 5% fetal calf serum. Both cell lines were plated in 100-mm plates at the same time (7×10^5 6-TG⁺ and 100 6-TG⁻), allowed to attach, and then treated with the TPA ($0.1 \mu\text{g ml}^{-1}$) or saccharin and 6-thioguanine ($10 \mu\text{g ml}^{-1}$). The medium was replaced after 3 days and 5 days with medium containing 6-thioguanine. After 7 days the colonies were fixed, stained with Giemsa and scored. The results are expressed as the percentage of plated 6-TG⁺ cells (determined by control plates containing 100 6-TG⁺ cells for each treatment) that formed colonies in the presence of the 6-TG⁻ cells. None of the saccharin doses decreased the colony-forming ability. All of these *in vitro* promotion assays are done with non-toxic concentrations of presumptive promoters.

above 2 mg ml^{-1} , there is a linear 'rescue' of 6-thioguanine resistant cells. Compared with TPA, the effect of saccharin on the inhibition of metabolic cooperation is weak. Whereas $0.1 \mu\text{g ml}^{-1}$ of TPA resulted in a four- to fivefold increase in the recovery of 6-thioguanine resistant cells, 5 mg ml^{-1} of saccharin caused only a twofold increase.

Since Wolff and Rodin⁴ showed that both impure (lot S1022, Maumee process, Sherwin-Williams) and pure samples of saccharin induced sister chromatid exchanges, we performed an experiment to find whether this same impure saccharin and a commercial preparation (Sweet'N Low) would affect metabolic cooperation. Table 1 shows that both the impure and pure saccharin worked to block metabolic cooperation. Sweet'N Low failed to block metabolic cooperation, probably because only 4% of the mixture was saccharin (the maximum concentration of saccharin was 0.2 mg ml^{-1}).

The demonstration that the saccharin preparation seems to be a weak promoter of certain cancers and to block metabolic cooperation. Table 1 shows that both the impure and pure to other promoters suggests that, at least for rodents, it can influence tumorigenesis at a high concentration by affecting cell-cell communication rather than by mutating cells. The observation that sister chromatid exchanges are induced not only by known DNA damaging agents/mutagens²⁴, but also by a tumour promoter (TPA)²⁵ and anti-promoters raises some new and interesting problems related to both the mechanism of action of tumour promoters and the assays used to detect them.

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Table 1 Recovery of 6-thioguanine resistant cells in the presence of TPA, pure saccharin, impure saccharin and Sweet'N Low

No. of V79 cells (HG-PRT ⁺)	No. of 6-TG ⁻ cells (HG-PRT ⁻)	Drug	Concentration	% Recovery
—	100	—	—	79.1
7×10^5	100	—	—	18.9
7×10^5	100	TPA	$0.1 \mu\text{g ml}^{-1}$	96.4*
7×10^5	100	Pure saccharin	3 mg ml^{-1}	40.2*
7×10^5	100	Impure saccharin	3 mg ml^{-1}	42.0*
7×10^5	100	Sweet'N Low	3 mg ml^{-1}	22.2
7×10^5	100	Sweet'N Low	5 mg ml^{-1}	23.6

* Treatment significantly increased recovery ($P < 0.01$) according to SNK test.

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Embryonal carcinoma stem cells lack a function required for virus replication

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Embryonal carcinoma (EC) stem cells are thought to be equivalent to the ectoderm of the preimplantation mouse embryo because they share morphological, biochemical and antigenic properties^{1,2}. These near diploid EC cells can be induced to differentiate into cell types of all three germ layers^{1,2}. They also can take part in normal differentiation and contribute cells to many tissues in chimaeric mice derived from preimplantation mouse embryos injected with them³. The ability to restrict the growth of certain viruses is a distinguishing characteristic of EC cells^{4–8}, but it is intriguing that this restriction disappears after differentiation. The ability of EC cells to block totally productive infection by the highly infectious and oncogenic Moloney strain of murine leukaemia virus (M-MuLV) is remarkable, as virtually all other murine cells are permissive to the growth of this virus. Teich *et al.*⁷ suggested that replication of M-MuLV in EC cells is blocked at some point after integration of proviral DNA into the cell's genome. Here I enquire into the dominant or recessive nature of the restriction, that is, do EC cells show transdominant inhibition of virus replication analogous to that imposed by interferon or the mouse *Fv-1* locus, or is the block to MuLV replication due to the lack of something in the EC cell? This report shows that the restriction of M-MuLV in EC cells can be overcome by fusion with permissive cells. The resulting complemented growth of M-MuLV in EC/permissive cell heterokaryons indicates that the restriction over M-MuLV is a recessive trait of EC cells and, therefore, defines a hitherto unknown host function that is vital to virus growth but missing in EC cells.

Virtually all permissive mouse cells (SC-1 and 129/Sv mouse embryo fibroblasts (MEF)) infected with M-MuLV formed syncytial plaques in an infectious-centre XC assay (Table 1a). Infected EC-A cells did not form plaques, indicating that pro-

geny virus was not produced. However, when EC-A cells were infected with M-MuLV and then fused with uninfected permissive cells, XC plaque formation was evident (Table 1b). This complementation of M-MuLV growth in infected EC-A cells was provided by fusion with uninfected SC-1 cells, 129/Sv secondary MEF, mink lung cells or rabbit epithelial cells. The latter two types complement M-MuLV growth although these mink and rabbit cells are themselves resistant to infection by M-MuLV (either because they lack surface membrane receptors⁹ or because the virus cannot penetrate their plasma membranes). In the control, fusion of infected EC-A cells with uninfected EC-A cells did not result in XC plaque formation. The appearance of XC plaques did not result from free or EC-bound infectious virus because the addition of permissive cells to infected EC-A cells, without fusion, resulted in little, if any, plaque formation. Also, culture supernatants collected at various times before and after polyethylene glycol (PEG) fusion, and used to 'infect' SC-1 cells, were consistently negative for infectious plaque-forming virus. This was done to eliminate the

Table 1 Complementation of M-MuLV growth in EC-A cells

M-MuLV-infected cells*	Uninfected complementing cells	log ₁₀ ICPFU ml ⁻¹ †	
		fused‡	not fused
<i>a</i>			
EC	—	<0.3	(<0.3)
SC-1	—	4.1	(5.4)
129/Sv MEF	—	3.6	(4.9)
RLE	—	<0.3	(0.6)
MLC	—	<0.3	(<0.3)
<i>b</i>			
EC	EC	<0.3	(<0.3)
EC	SC-1	3.1	(1.4)
EC	129/Sv MEF	2.6	(0.6)
EC	RLE	1.9	(<0.3)
EC	MLC	1.7	(<0.3)

*EC-A cells were supplied by Dr W. C. Speers as a subclone²⁵ of the PCC4azal embryonal carcinoma cell line derived from a teratocarcinoma of the 129/Sv mouse, as first described by Jakob *et al.*¹⁹. When grown as monolayers *in vitro* without feeder cells, less than 0.1% of these cells spontaneously differentiate without specific stimulation; PCC4azal and most other subclones spontaneously differentiate at higher frequencies. EC-A cells were grown in Eagle's minimal essential medium (EMEM) containing 10% fetal calf serum and antibiotics. The SC-1 murine cell line is highly permissive to type-C murine retrovirus infection²⁰. 129/Sv MEF were secondary cells derived from 14-day embryos of a 129/Sv mouse. Rabbit lung epithelial (RLE) and mink lung cells (MLC)²¹ are permissive for xenotropic and amphotropic viruses and non-permissive to ecotropic MuLV. M-MuLV is the exogenous Moloney strain of MuLV; the plaque-forming M-MuLV-148 subclone used in these studies has a ecotropic host range and grows well in mouse cells but very little, if at all, in cells from other species.

† Virus production was assayed by a quantitative infectious-centre XC plaque assay, SXC, that requires only a single cycle of infection in mouse cells²². Therefore, a single virus-producing mouse cell caused an XC syncytial-plaque expressed as an infectious-centre plaque forming unit (ICPFU). Cells were treated with DEAE dextran (25 µg ml⁻¹ for 30 min) before infection with M-MuLV at a multiplicity of 1–20 plaque forming units per cell. EC cells infected in this manner were allowed to adsorb virus for 1 h at 37 °C. The cells were then washed twice with EMEM and incubated for an additional 1–2 h to allow penetration of the cells by the adsorbed virus. The infected cells were lifted and washed extensively to ensure the elimination of any free M-MuLV that did not adsorb to the cells. These infected EC cells, as a single cell suspension, were plated on 35-mm diameter tissue culture plates at 2 × 10⁵ cells per plate, and an equal number of complementing cells was added. The cells were allowed to attach to the surface of the dishes for at least 2 h before fusion.

‡ Cells were fused with 47.5% polyethylene glycol (PEG) as described previously²³. The efficiency of cell fusion and the toxicity of PEG to cells is difficult to estimate as these factors vary from one cell type to another and are highly dependent on PEG concentration, which can vary throughout an exposed plate due to the presence of residual media²³. Cultures that were observed 3 h or more after PEG exposure had areas of non-viable cells, areas of large multinucleated heterokaryons, and areas of single cells and small heterokaryons. Viable cells (trypan blue exclusion) at 16 h after infection normally contained approximately 10% bi- and multinucleated heterokaryons. Multinucleated heterokaryons were lost from cultures that were carried for several generations. Similar results were obtained when cells were fused with inactivated Sendai virus, although there were fewer multinucleated heterokaryons formed and little if any toxicity. Fused or unfused infected cells were lifted 16 h after infection, before progeny virus-induced secondary infections could occur, counted, and dilutions of the single cell suspensions were cocultivated with XC cells and dexamethasone, as described²⁴. Heterokaryons that produced progeny virus caused infection and fusion of surrounding XC cells resulting in syncytial plaques that were small relative to M-MuLV infected permissive mouse cells, suggesting that fewer progeny virus were produced by the fused cells.

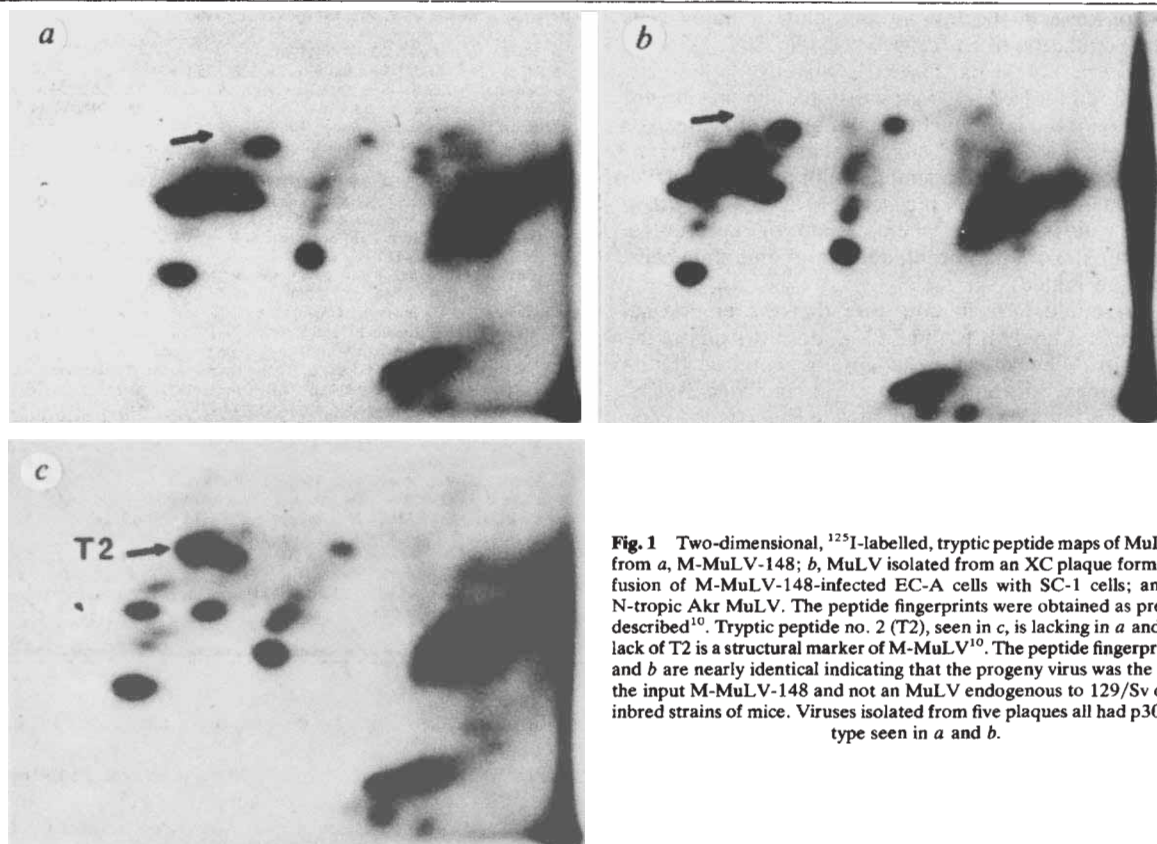


Fig. 1 Two-dimensional, ^{125}I -labelled, tryptic peptide maps of MuLV p30s from *a*, M-MuLV-148; *b*, MuLV isolated from an XC plaque formed after fusion of M-MuLV-148-infected EC-A cells with SC-1 cells; and *c*, an N-tropic Akv MuLV. The peptide fingerprints were obtained as previously described¹⁰. Tryptic peptide no. 2 (T2), seen in *c*, is lacking in *a* and *b*. This lack of T2 is a structural marker of M-MuLV¹⁰. The peptide fingerprints of *a* and *b* are nearly identical indicating that the progeny virus was the same as the input M-MuLV-148 and not an MuLV endogenous to 129/Sv or other inbred strains of mice. Viruses isolated from five plaques all had p30s of the type seen in *a* and *b*.

possibility that plaques were caused by secondary infections of SC-1 cells by input M-MuLV liberated from EC cells after exposure to PEG.

Viruses were isolated from individual XC plaques and grown in SC-1 cells, after which peptide maps of the viral p30 core protein were obtained, as described previously¹⁰. Figure 1 shows that the p30 peptide maps contained markers specific for M-MuLV, indicating that the plaques were caused by progeny of the input M-MuLV, as opposed to a possible fusion-induced production of virus endogenous to the EC-A^{11,12} or permissive cells. Therefore, heterokaryons formed between infected EC-A cells and uninfected permissive cells produced infectious progeny M-MuLV, whereas infected EC-A cells alone did not.

The efficiency of permissive cell complementation of M-MuLV growth in EC cells was dependent on the time of heterokaryon formation. M-MuLV-infected EC-A or F-9 cells were fused with uninfected SC-1 cells at various times after infection and assayed for infectious centres. As seen in Table 2, complementation of M-MuLV growth in EC cells was much more frequent when heterokaryons were formed 8–12 h after infection. Unexpectedly, before this time, and even after, fusion yielded little complementation of M-MuLV growth. These results suggest: (1) that the virus requires the host cell function only during a discrete period of its replication cycle, that is 8–12 h after infection; and (2) as heterokaryon formation before 8 h postinfection was not efficient in complementing M-MuLV growth, the host function must itself be labile and not replenished in the heterokaryon. Since the entire contents of the EC and permissive cells are present initially in heterokaryons, a dominant regulatory component of the EC cell apparently represses production of the required function that permissive cells express. This is not surprising when one considers the ability of EC cells to confer pluripotency to somatic cell hybrids between EC cells and thymocytes¹³ or Friend erythroid cells⁸ and suggests a generalized dominant nature of undifferentiated embryonic stem cells.

A separate, third, consideration is that complementation of M-MuLV production in infected EC-A and F-9 cells, by fusion

with uninfected SC-1 cells, decreases rapidly 12 h or more after infection (Table 2). After this interval, virus apparently cannot use the host component that complements M-MuLV growth in EC/permissive cell heterokaryons. This suggests that the virus has changed or is under different control after the period of complementation passes (8–12 h postinfection). We have isolated subclones of infected EC cells that are known to be non-productively infected with M-MuLV¹⁴. These cells cannot be induced to produce M-MuLV by fusion with permissive cells. This latter point is consistent with related studies with M-MuLV⁷ and SV40¹⁵, in which EC/permissive cell heterokaryons, formed several days after infection of EC cells, did not replicate virus, whereas fusion soon after polyoma virus infection did result in viral antigen expression¹⁶. Also, as complementation decreased when fusion occurred later than 12 h postinfection, it is unlikely that fusion simply allowed

Table 2 Temporal effect of SC-1 cell complementation of M-MuLV growth in EC-A and F-9 cells

Expt	EC cell	ICPFU per plate*						
		No PEG	4	8	12	16	20	24
1	EC-A	2	86	162	248	12	ND	0
2	EC-A	1	20	98	124	ND	3	ND
3	EC-A	0	0	14	40	12	ND	0
4	EC-A	8	70	860	787	86	12	ND
5	EC-A	26	112	690	1274	ND	118	ND
6	EC-A	1	30	110	114	ND	5	ND
6	F-9	0	37	180	228	ND	20	ND

*Subconfluent EC-A and F-9 monolayer cells were infected with M-MuLV, co-cultivated with SC-1 cells, fused with PEG, and assayed for XC plaque formation as described in Table 1. F-9 cells, a 'nullipotent' EC cell line that does not spontaneously differentiate in monolayer culture²⁶ were from Dr F. Dutko. F-9 cells were grown in monolayers on plastic tissue culture flasks or plates that had been previously treated with 0.1% gelatin at 4 °C for 2 h to enhance attachment. PEG fusion was timed from the first exposure of EC cells to M-MuLV. Numbers represent the average ICPFU for duplicate or triplicate plates at each time point. ND, Not determined.

amplification of virus in the few infected differentiated cells present in the population of EC cells.

These studies identify the existence of a missing factor in EC cells that is required for M-MuLV growth, although they do not specify the nature of the host function, possibly an enzyme(s) or organelle(s). Recent studies^{17,18} suggest that the undifferentiated F-9 line of EC cells may be defective in producing stable SV40 RNA transcripts. Whether the lack of M-MuLV growth in F-9 and EC-A cells is a similar phenomenon, or represents a general characteristic of undifferentiated embryonic stem cells, has yet to be determined.

The data presented here demonstrate that the embryonal carcinoma stem cell lines, F-9 and EC-A, do not support the growth of M-MuLV because they lack a host function that is required for virus replication and must be available 8–12 h postinfection. This function can be supplied to EC cells by fusion with differentiated mouse, rabbit or mink cells. If this fusion-induced complementation of M-MuLV growth can be used to purify the responsible factor from permissive cells, the related function should become clear.

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Encephalomyocarditis virus infection of cultured murine pancreatic β -cells

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Encephalomyocarditis virus (EMC) possesses many of the biological and pathogenetic features of the human Group B coxsackieviruses which have been implicated in the aetiology of insulin-dependent diabetes mellitus^{1,2}. The M variant of EMC produces a diabetes-like disease when inoculated into some strains of mice. In these animals, cytolysis of β -cells is prominent, and there is an accompanying mononuclear inflammatory cell response (insulitis). Hyperglycaemia is observed during the acute stages of infection followed by varying degrees of carbohydrate intolerance^{3–5}. In other mouse strains, β -cell lesions are less prominent and insulitis fails to occur, even though the animals develop a systemic infection. In these animals diabetes is observed either infrequently or not at all. Although the susceptibility of mice to the diabetogenic effect of the virus is believed to be influenced by one or more recessive genes, the pathogenetic basis for differences between the strains has not been defined^{6,7}. The basis for the unique tropism of the M variant for β -cells also is uncertain because other serotypically similar strains of this virus cause pancreatic acinar cell necrosis, but lack a diabetogenic effect^{3,5}. We have studied mouse pancreatic β -cells in tissue culture to determine whether or not these cells are uniquely susceptible to the M variant of EMC. Additionally, we examined the viral susceptibility of cultured β -cells derived from mouse strains that varied in their resistance to the diabetogenic effects of the M variant. The results show that cultured mouse β -cells can be infected by a variety of EMC viruses, and β -cells from different strains of mice are susceptible to infection by the M variant of EMC.

The majority of cells in our monolayer cultures stained positively by an immunoperoxidase technique^{8,9} using anti-bovine insulin serum. The cultures secreted insulin into the medium at a gradually increasing rate for periods of longer than 1 month^{10,11}. However, because the monolayers were prepared from fresh pancreata, the number of cells and the absolute amount of

insulin released into the culture dishes varied from experiment to experiment.

After maintenance for 9 days *in vitro*, monolayers were infected with 10^4 plaque-forming units of virus at an estimated multiplicity of infection of ~ 1 . The deviation and properties of the M variant and the non-diabetogenic E (ref. 12), r^+ (ref. 13), and Mengo¹⁴ strains have been reported¹⁵. The viruses were

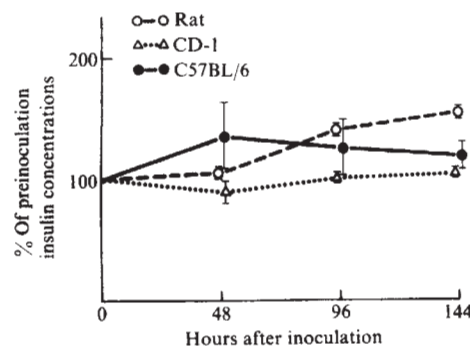


Fig. 1 Monolayer cultures of mouse β -cells were prepared from the pancreatic tissue of 4- to 6-week-old mice. The isolated pancreata were dissociated in a phosphate-buffered saline solution containing 0.04% collagenase, 0.02% α -chymotrypsin, 0.03% DNase and 4% fetal calf serum. Rat β -cells were prepared by the method of Chick *et al.*¹¹. β -Cell preparations were enriched using discontinuous Ficoll gradients and purified by differential adhesion and treatment with cysteine-free culture medium²⁴. Cells were plated into multiwell plates (24 wells per plate, Co-Star) and maintained with Medium 199 (Gibco) containing 10% fetal calf serum, glucose (300 mg dl⁻¹) and gentamicin (1 mg dl⁻¹). The culture medium was replenished at 48-h intervals. Insulin in the culture medium was determined by the method of Wright *et al.*²⁵ using I¹²⁵-labelled porcine insulin (Cambridge Nuclear) diluted with cold porcine insulin (Dr Ronald Change, Eli Lilly), and guinea pig anti-bovine insulin serum (Linco) with purified mouse insulin (Novo) serving as a standard. Using this method insulin concentrations as low as 50 μ U per ml of culture medium can be detected. Insulin release by β -cells in control cultures is displayed. The cultures were prepared from the pancreata of CD-1 and C57BL/6 mice and CD rats (Charles River). Data are expressed as a percentage of the insulin released during days 7 to 9 of culture. The 100% insulin concentrations averaged 1.5 $\pm$ 0.3 mU per culture for the CD-1 cultures, 0.5 $\pm$ 0.03 mU per culture for the C57BL/6 cultures and 10.6 $\pm$ 0.4 mU per culture for the CD rat cultures.

grown and titred in a continuous line of mouse fibroblasts (L-929) maintained in this laboratory.

Culture medium was collected at 48-h intervals after infection and assayed for insulin. Control cultures released insulin at a steady rate (Fig. 1). The results of insulin radioimmunoassays on the medium of infected β -cell cultures prepared from CD-1 mice are summarised in Fig. 2. Studies with the diabetogenic M and the non-diabetogenic E, r^+ and Mengo strains were conducted in a similar fashion. In replicate experiments insulin in the culture medium was reduced appreciably at 48 h after inoculation and was not detectable after 96 h. During this time cultures were monitored daily by phase contrast microscopy, and extensive cytolysis was observed. Thus, morphological evidence of cell death clearly supported the insulin data. The effects noted above were not influenced substantially by virus dosage. To exclude nonspecific cytotoxic effects of the viral inoculum and subtle changes in culture conditions, parallel studies were carried out with β -cell cultures prepared from neonatal rats. Insulin release by these cells was unchanged during the course of the experiments, and morphological alterations were not observed. Although it is possible that the procedure for the preparation of cultures altered mouse, but not rat, β -cells in such a way as to unmask hidden virus receptors, we feel this is unlikely. The 9-day culture period before infection should be of sufficient duration to permit repair of membrane components¹⁶.

The results of experiments using β -cell cultures prepared from diabetes-prone CD-1 and resistant C57BL/6 mice are shown in Fig. 3. Radioimmunoassays on the medium from these cultures demonstrated a comparable decline in insulin release, and similar cytopathology was observed.

It has been contended in other studies that genetic factors code for the presence of viral receptor density on the β -cell surface and thus determine susceptibility to the virus^{17,18}. The morphological methods used by these investigators to identify β -cells are questionable, however, due to nonspecific staining^{9,19}. If the cell cultures in their studies comprised a mixture of fibroblasts and pancreatic cells (acinar and insular), it seems likely that the assays could not assess definitively the receptor density of β -cells. Using insulin release as a measure of

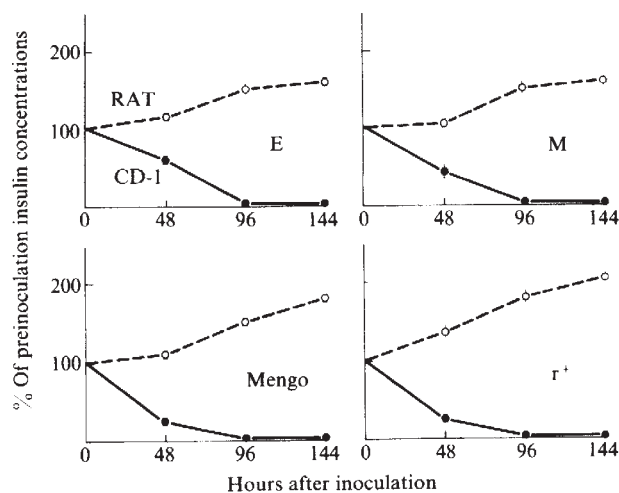


Fig. 2 Insulin release by β -cells in culture after inoculation with various strains of EMC virus. The cultures were prepared from the pancreata of CD rats and CD-1 mice. Data are expressed as a percentage of the insulin released in each culture during the 48-h period immediately preceding inoculation (mean $\pm$ s.e.m. of four observations). The average 100% pre-inoculation insulin concentrations for the CD rat and CD-1 mouse cultures, respectively, are: 8.6 ± 0.6 mU per culture and 1.5 ± 0.1 mU per culture (E variant); 10.2 ± 0.3 mU per culture and 1.5 ± 0.1 mU per culture (M variant); 9.5 ± 0.3 mU per culture and 1.3 ± 0.07 mU per culture (Mengo); and 7.9 ± 1.5 mU per culture and 1.4 ± 0.03 mU per culture (r^+).

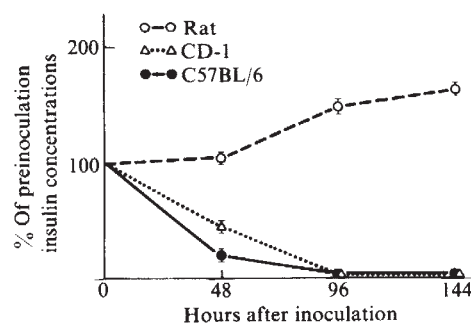


Fig. 3 Insulin release by β -cells in culture after inoculation with EMC M variant virus. The cultures were prepared from the pancreata of CD rats, CD-1 mice and C57BL/6 mice. Data are expressed as a percentage of the insulin released in each culture during the 48-h period immediately preceding inoculation (mean $\pm$ s.e.m. of four observations). The average 100% pre-inoculation insulin concentrations are: 10.2 ± 0.3 mU per culture (CD rat); 1.5 ± 0.1 mU per culture (CD-1 mouse); and 0.5 ± 0.02 mU per culture (C57BL/6 mouse).

viral induced cell injury, we found no difference between β -cells from mice resistant and susceptible to the diabetogenic effect of the virus. Moreover, several antigenically similar, but non-diabetogenic, EMC virus strains infected β -cells in culture. Thus, β -cells *in vitro* fail to exhibit unique susceptibility for the M variant.

Recent *in vivo* studies in our laboratory indicate that the islets of resistant mice sustain significant cytological injury. Although pancreata are depleted of insulin during the acute stages of infection, hyperglycaemia fails to develop. We have also found that alterations in the metabolic state of mice affect the severity of β -cell damage and the expression of diabetes (refs 20, 21 and B.J.D.A., G.L.W. and J.E.C., unpublished data). Similarly, culture conditions could affect susceptibility of β -cells to this virus^{22,23}. Our observations do not exclude the possibility that viral susceptibility of β -cells in the intact animal is a consequence of the presence of specific receptors. We suggest, however, that other factors such as metabolic influences also are critical determinants affecting viral susceptibility and injury to β -cells.

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Non-mendelian mutation affecting ribulose-1, 5-bisphosphate carboxylase structure and activity

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Chlamydomonas reinhardtii, a haploid unicellular green alga, is the only organism in which the non-mendelian (uniparental) genes thought to reside in chloroplast DNA have been observed to recombine. Thus, genetic mapping by recombination analysis is possible, and a single linkage group with a large number of markers has been characterized^{1,2}. Although circumstantial evidence is consistent with the hypothesis that this uniparental linkage group is located in the chloroplast,^{1,2} there is no direct evidence associating any of the known markers with chloroplast DNA. As the gene for the large subunit (LS) of ribulose-1, 5-bisphosphate carboxylase (RubPCase) has been located on the physical map of *C. reinhardtii* chloroplast DNA,^{3,4} a genetic marker in this same gene would help to prove the relationship between the uniparental linkage group and chloroplast DNA. We have now succeeded in isolating a mutation in a *C. reinhardtii* gene which controls the structure and function of the LS of RubPCase. This new genetic marker will serve as the first reference point in our efforts to correlate the physical and genetic maps for the chloroplast genome. At the same time, the fact that we have obtained this mutant should encourage further attempts to produce genetic modifications of this key photosynthetic enzyme with the goal of improving plant productivity⁵.

RubPCase is an oligomeric enzyme which has two important enzymatic activities. The holoenzyme has eight copies of the 55,000 molecular weight MW LS and eight copies of a 12–20,000 MW small subunit (SS) which is a nuclear gene product⁶. Net CO₂ fixation during photosynthesis depends on the RubPCase-catalysed carboxylation of ribulose-1, 5-bisphosphate in the Calvin cycle⁷. On the other hand, the enzyme also catalyses the reaction of O₂ with the same substrate. This reaction, which is the first step in photorespiration, leads ultimately to CO₂ evolution and an apparently wasteful consumption of Calvin cycle intermediates which might otherwise be used for CO₂ fixation⁷. The catalytic site, which seems to be the same for both the carboxylase and oxygenase activities⁸, is located on the LS⁷.

Non-photosynthetic mutants of *C. reinhardtii*, including presumptive LS mutants, can be maintained on medium containing acetate. Many such mendelian⁹ and also uniparental¹⁰ acetate-requiring mutants have been isolated, but only one has been found with a specific defect in a Calvin cycle enzyme (phosphoribulokinase)¹¹. We reasoned that a Calvin cycle mutant, in addition to having an acetate-requiring phenotype, might be photosensitive as a result of the build-up of unused reductant generated by an active photosynthetic electron transport system. A light-sensitive phenotype would also account for the apparent rarity of RubPCase mutants, and Calvin cycle mutants in general, because selection experiments have usually been carried out at high light intensities. Taking this into account, we adopted a mutant selection protocol which would allow the recovery of light-sensitive, acetate-requiring mutants.

Independent cultures of wild-type *C. reinhardtii*, strain 2137 mt⁺ (ref. 12), were grown in 10 mM sodium acetate medium in the dark at 25°C until they reached stationary phase. To enhance uniparental mutant recovery, 1 mM 5-fluorodeoxyuridine was included in these cultures¹³. Cells were then collected, washed by centrifugation and mutagenised with ethyl methanesulphonate¹⁴. Washed cells were grown in the dark in acetate medium to allow expression of mutant phenotypes. Cells from each of these cultures were plated at 100–500

cells per plate onto acetate medium in the dark. Colonies were replica plated to medium without acetate and placed in 4,000 lx cool white fluorescent light. Colonies unable to grow on this minimal medium were recovered at a frequency of one per 10³ cells plated, and included both mendelian and uniparental acetate requirers, as determined by standard tetrad analysis¹⁵. All the uniparental mutants were light sensitive, growing better in the dark with acetate than in the light (2,000 lx) with acetate.

RubPCase was purified from all the uniparental mutants by sucrose gradient centrifugation¹⁶ and subjected to isoelectric focusing (IEF) in 8 M urea¹⁷. Molecular weights were determined by two-dimensional SDS-polyacrylamide gel electrophoresis¹⁷ and used to verify the identity of the RubCase LS and SS bands observed on the first-dimension IEF gels^{12,18}.

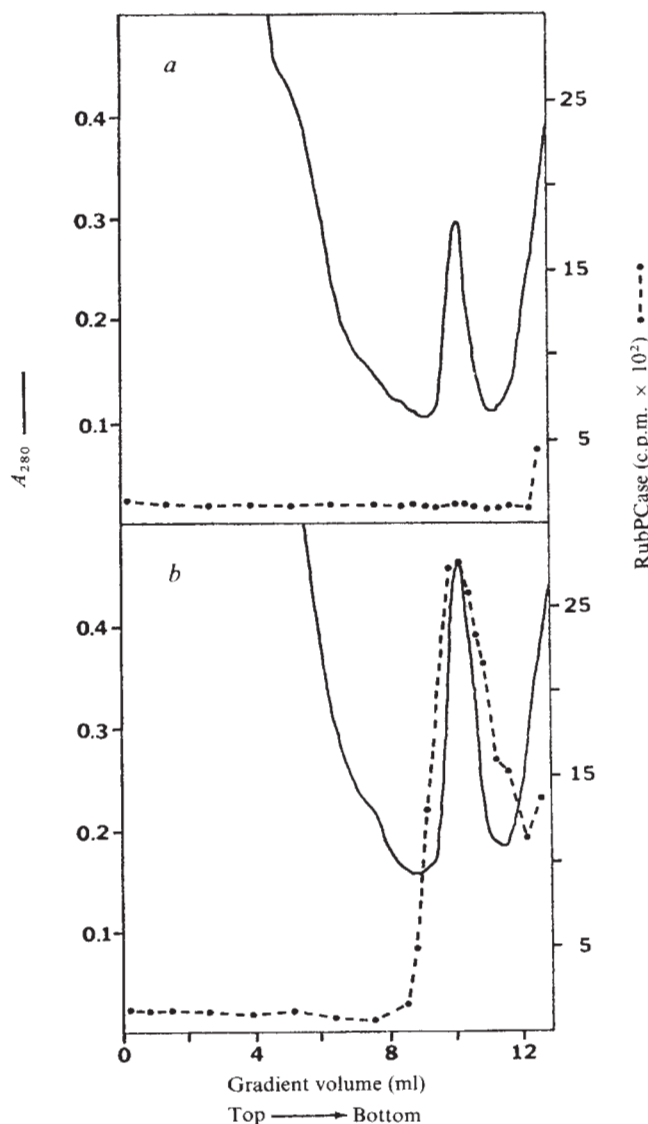
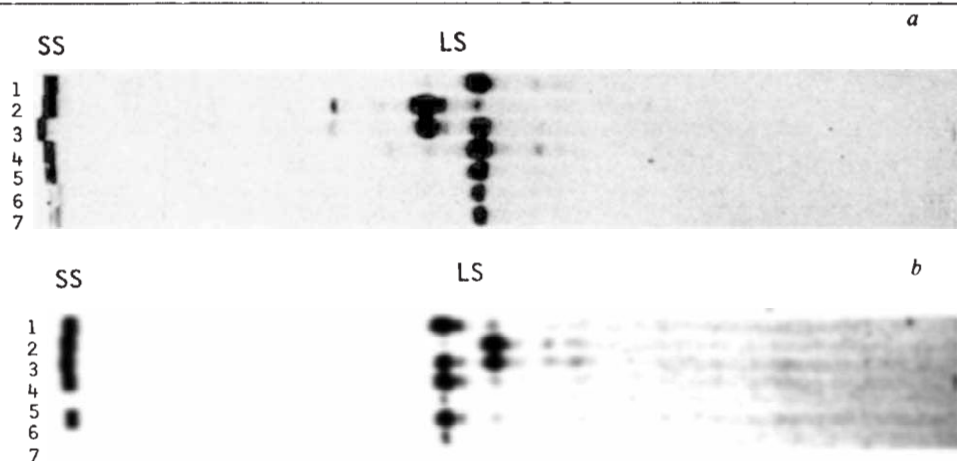


Fig. 1 Sucrose gradient centrifugation and assay of RubPCase from *rcl-u-1* and wild type. *a*, *rcl-u-1-10-6C*; *b*, wild type. Cultures were grown in acetate medium in the dark. Approximately 6×10^8 cells were sonicated at 0°C in 1.5 ml of sonication buffer: 1 mM dithiothreitol (DTT), 5 mM MgCl₂, 10 mM NaHCO₃ and 50 mM Tris base, pH 7.5. Following centrifugation at 27,000g for 15 min, 1 ml of the supernatant was layered onto a 12-ml linear sucrose gradient (10–30% sucrose in sonication buffer), which was run in an SW-40 rotor at 37,000 r.p.m. for 20 h at 4°C. The gradient was fractionated into 0.3-ml fractions and scanned at 280 nm. RubPCase was assayed¹⁶ by mixing 0.1 ml of a sucrose gradient fraction with 0.1 ml of reaction mix: 1 mM DTT, 15 mM MgCl₂, and 90 mM NaH¹⁴CO₃ (0.22 μCi μmol⁻¹). After 15 min at 25°C, the reaction was terminated with the addition of concentrated HCl/acetic acid (4:1) and the samples were then processed for liquid scintillation counting.

Fig. 2 IEF of RubPCase from reciprocal crosses between *rcl-u-1* and wild type. *a*, *rcl-u-1* mt⁺ × wild-type mt⁻: (1) *rcl-u-1* mt⁺; (2) wild-type mt⁺; (3) mixture of (1) and (2); (4)–(7) progeny from a single tetrad. *b*, Wild-type mt⁺ × *rcl-u-1* mt⁻: (1) wild type mt⁺; (2) *rcl-u-1* mt⁻; (3) mixture of (1) and (2); (4)–(7) progeny from a tetrad. Mendelian markers present in the crosses segregated 2:2 among the progeny. The SS on gels B5 and B7 did not reproduce well in the photograph. All parents and progeny were grown in acetate medium in the dark. RubPCase was purified by sucrose gradient centrifugation (Fig. 1). Samples obtained from the gradient were prepared by adding solid urea to 9M, and applied to 13.5 × 0.4 cm gels as 50-μl aliquots (5–50 μg RubPCase). IEF was carried out¹⁷ with pH 5–7 ampholines and 8M urea in polyacrylamide gels for 12 h at 400 V and 1 h at 800 V constant voltage at 25 °C. The gels were stained in bromophenol blue and Coomassie blue. The pH gradient was measured with stab pH and reference electrodes on fresh gels. This showed that the isoelectric point of the SS was 7.4 and that of the wild type LS was 6.3.



One mutant was found to have a lower isoelectric point for the LS. This mutant, named *rcl-u-1-10-6C* (*rcl-u-1* referring to its genetic locus), was a stringent acetate requirer that could not be maintained on acetate medium above 2,000 lx, but grew as well as wild type in the dark. Although *rcl-u-1-10-6C* maintained normal levels of RubPCase holoenzyme (Table 1), RubPCase activity was absent (Fig. 1). Further biochemical analysis (Table 1) showed that the mutant had normal levels of photosystem II (PSII) activity and chlorophylls, but lacked the ability to fix CO₂. The mutant was indistinguishable from wild type in holoenzyme sedimentation value (Fig. 1) and in LS and SS MW (data not shown). Reciprocal crosses indicated that the altered LS was inherited in a uniparental pattern (Fig. 2), along with the light-sensitive, acetate-requiring phenotype.

The evidence presented suggests that *rcl-u-1-10-6C* is a mutation in the chloroplast structural gene of the LS. Unlike other presumptive RubPCase mutants^{19,20}, the mutant phenotype is inherited in a uniparental pattern, which is the pattern expected for a mutation in a chloroplast gene^{21,22}. Because the mutant maintains normal PSII activity, amount of chlorophyll, and amount of RubPCase protein, defects in chloroplast protein synthesis or other defects which exert pleiotropic effects can be

ruled out. This is not the case for several maternally inherited mutants of *Oenothera* which have been presumed to be LS structural gene mutants²³. In contrast to other presumptive RubPCase mutants^{19,20,23,24} *rcl-u-1-10-6C* is the first mutant in which a change in LS structure is evident. Because apparently normal holoenzyme structure exists in the mutant, major processing errors are probably not responsible for the LS alteration. The difference in IEF properties between the mutant and wild-type LS could be due to a difference in primary amino acid sequences, or a difference in secondary modification. The critical discrimination between these two possibilities requires comparative sequence analysis of the two proteins. In either case, *rcl-u-1* is a uniparental gene which controls the apparent charge and activity of the RubPCase LS. We are now determining whether this gene is linked to the other uniparental genes in *C. reinhardtii*.

The photosensitivity of *rcl-u-1-10-6C* must be considered in designing protocols for obtaining additional mutants. Light intensities in excess of 2,000 lx, as have been used by other workers¹⁰, would not allow this mutant to survive. We have not investigated the mechanism of photosensitivity in this mutant, but we know that light-sensitive phenotypes are not confined to mutants with Calvin cycle defects. We have recovered many light-sensitive, non-photosynthetic mutants with specific defects either in PSI or in PSII. Each group includes both mendelian and uniparental mutants, which will be discussed in detail elsewhere.

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Table 1 Biochemical comparison of *rcl-u-1-10-6C* and wild type

	<i>rcl-u-1-10-6C</i>	Wild type
CO ₂ fixation (μmol per h per mg Chl)	12	640
PSII activity (DCPIP) (μmol per h per mg Chl)	135	144
Chlorophyll <i>a</i> + <i>b</i> (Chl) (mg per 1 × 10 ⁹ cells)	1.11	1.30
RubPCase protein (mg per 1 × 10 ¹⁰ cells)	4.39	4.36

Cultures were grown in acetate medium in the dark. CO₂ fixation was assayed by adding 0.2 ml of 50 mM NaH ¹⁴CO₃ (0.5 μCi μmol⁻¹) to 2 × 10⁷ cells in 2 ml of minimal medium at 25 °C and 60,000 lx (ref. 12). For PSII activity, cells were sonicated at 4 °C in reaction buffer: 2.5 mM MgCl₂, 20 mM KCl, 1 mM K phosphate buffer (pH 7.0)²⁵. The lysate was centrifuged at 480g for 5 min and the supernatant was diluted 10× with 0.1 mM dichlorophenol indophenol (DCPIP) in reaction buffer. The red light-dependent (180 μE m⁻² s⁻¹ between 640 and 740 nm) reduction of DCPIP was measured spectrophotometrically at 25 °C. Chlorophyll (Chl) was measured spectrophotometrically in 80% acetone extracts of whole cells²⁶. RubPCase protein was estimated from the absorbance at 280 nm of the RubPCase peak on sucrose gradients¹⁶.

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MATTERS ARISING

A 'random transition' in the cell cycle?

EVIDENCE is presented by Shields¹, partly mathematical and partly from real and simulated experiments, that the cell cycle contains an exponentially distributed phase. Unfortunately, there are flaws in his probabilistic argument at two points. His conclusions are probably not invalidated, but it seems appropriate to point out the errors in the hope that they will not be repeated elsewhere.

We are concerned with the probability distribution of cell cycle times, and with the distribution of the difference in cycle time of sister cells. It seems that sister cycle times T_1 , T_2 , say, may be well represented in terms of three independent random variables T_B , U_1 , U_2 as

$$\begin{aligned} T_1 &= T_B + U_1 \\ T_2 &= T_B + U_2 \end{aligned} \quad (1)$$

Here, U_1 and U_2 are identically distributed, whereas T_B is common to the two sisters and must be a non-constant random variable for T_1 and T_2 to be correlated. Shields himself mentions that the T_i values are correlated, quoting a typical value for the correlation coefficient of 0.78; according to representation (1) above, the correlation is in general

$$\rho(T_1, T_2) = \frac{\text{Var}(T_B)}{\text{Var}(T_B) + \text{Var}(U_1)} \quad (2)$$

In contradiction to this, Shields then assumes that T_1 and T_2 are independent, in order to derive an expression for $\beta(t)$, the survivor function of $|T_1 - T_2|$, in terms of $Q(t)$, that of T_i . In fact, this argument may be rescued: in view of representation (1), $|T_1 - T_2| = |U_1 - U_2|$. On the basis of empirical α curves, we might assume the model

$$\begin{aligned} Q^*(t) &= \Pr\{T_i > t + T_B\} \\ &= \Pr\{U_i > t\} \\ &= \exp(-kt), \quad t \geq 0, i = 1, 2 \end{aligned} \quad (3)$$

from which it is true that

$$\begin{aligned} \beta(t) &= \Pr\{|T_1 - T_2| > t\} \\ &= \Pr\{|U_1 - U_2| > t\} \\ &= \exp(-kt), \quad t \geq 0 \end{aligned} \quad (4)$$

This approach is tantamount to redefining Shields' function $Q(t)$ as a conditional survivor function.

The second flaw concerns Shields' claim at the end of paragraph 5 that representation (4) implies representation (3). This

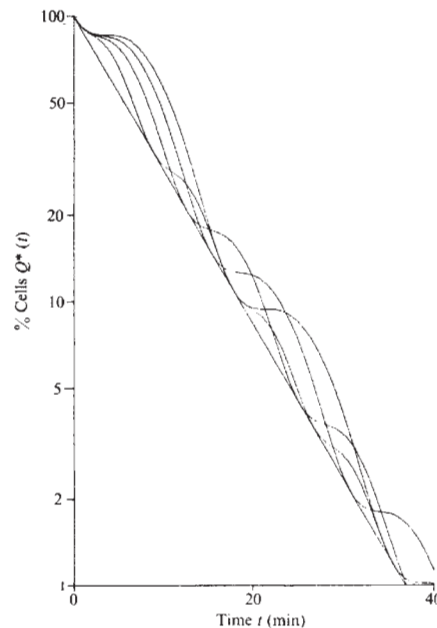


Fig. 1 Semi-logarithmic plot of $Q^*(t) = \left(1 + 2d^2 \sin^2\left(\frac{kt}{d}\right)\right) e^{-kt}$ against t for $k = \frac{1}{8}$ and $d^2 = 0, \frac{1}{8}, \frac{1}{4}, \frac{3}{8}$ and $\frac{1}{2}$.

is not the case, and many other forms of $Q^*(t)$ give the same expression for $\beta(t)$: for example, the whole family

$$\begin{aligned} Q^*(t) &= \left(1 + 2d^2 \sin^2\left(\frac{kt}{d}\right)\right) \times \\ &\quad \times \exp(-kt), \quad t \geq 0 \end{aligned} \quad (5)$$

as d varies from 0 (corresponding to representation (3)) to $1/2^{1/2}$. These solutions are illustrated in Fig. 1; presumably, however, they are rather unlikely to represent correctly the biological reality. Further, Shields' observation that exponential β curves are obtainable irrespective of the age of the younger of a sister pair is, of course, conclusive evidence that representation (3) is the correct solution, as such curves completely determine the joint distribution of (T_1, T_2) .

I thank Russell Smith for finding the counter-example (5) above.

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SHIELDS REPLIES—Green is quite right to point out that the cell cycle times of sister cells are frequently correlated and that my equation for the distribution of differences of sister cell cycle times is not valid without qualification. We have argued that correlations in the cycle times of sister cells arise because the cell cycle consists of two parts, one of which (the B phase) is common to the sisters, whereas the other (A state) is independently and identically distributed. According to this model, the correlation of cell cycle times of sister cells arises from the identity of the B phase, the differences from the A state. If these assumptions are correct, what does an exponential distribution of differences in cell cycle time (the β curve) tell us? Green points out that many A state distributions will give exponential curves (an example is given in his paper); this is why I added the condition that exponential β curves were obtained irrespective of the cell cycle times of the younger cell in the cell pair when curves are calculated from cohorted data.

If this condition is met, then, as Green pointed out, the A state must be exponentially distributed. However, if A states are not independently and identically distributed, other cell cycle models can be devised which give exponential β curves (D. Rigney, personal communication). What I had intended to do was to focus attention on the statistic $|T_1 - T_2|$, which has been largely ignored in cell cycle analysis. It should not be forgotten that any proposed cell cycle model should explain the exponential nature of $|T_1 - T_2|$ as well as give a description of the overall distribution of cell cycle times in a cell population. A modified version of the original transition probability hypothesis seems to do this successfully¹.

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BOOK REVIEWS

The road to equality

Eric Ashby

THE fear that Western civilization will collapse from exhaustion of resources was the Great Red Herring of the 1970s, deflecting our attention from a more likely cause of collapse: the unequal distribution of resources. In the 1980s we shall, I hope, get back to the problems that really matter, namely how to persuade — and, if we fail to persuade, compel — affluent nations to share their resources of food, stored energy and mineral wealth, with nations that lack one or other of these resources. If America and Canada cut down their acreage of grain crops, if the Arabs decide to keep their oil in the ground, if South Africa and Zimbabwe stop the export of chromium, the prime danger will not be privation; it will be war. The way ahead, in the short term at any rate, is now becoming clearer. It is not to pursue the mirage of zero growth, nor is it to let the market economy rip in a frenzy of free competition; it is to continue growth with prudence, cutting down waste, protecting the environment, and to distribute the products of growth with greater equity than nations have ever shown in the past.

This is the theme in what (to my mind) is the most important statement about the human condition that has been made in the last decade: the book of essays entitled *World Futures* produced from the Science Policy Research Unit in the University of Sussex (reviewed in *Nature* 276, 144-146; 1978). *World Futures* is a symposium by several authors. It does not ram a message down the reader's throat; it offers him the facts and leads him (with skilful encouragement by Marie Jahoda in the concluding chapter) to a choice among four options: high growth, high equality (a big cake fairly shared); high growth, low equality (a big cake hogged by the rich); low growth, high equality (a small cake fairly shared); and low growth, low equality (a small cake hogged by the rich). Marie Jahoda's choice is for the big cake fairly shared.

John Gribbin has written an epitome of *World Futures*. It is about half the length of the original book. One reason he gives for having written it is that *World Futures*

Future Worlds. By John Gribbin. Pp.225. (Sphere:London, 1979.) Paperback £1.75.

is "inevitably intimidating to the more casual reader". I'm afraid I don't like this reason. There are some issues — and this surely is one of them — on which people can't afford to be casual. If they will not take the trouble to read 390 pages of closely argued, but non-technical essays about the future, they have no right to hold opinions about the future.

But there are other reasons, and good ones, why John Gribbin wrote this book. First, he was associated with the team — though not a member of it — so his book is not just a rehash of *World Futures* with the title reversed: it is an observer's reflections on the work at Sussex. Second, he is an experienced journalist, skilled in the art of turning casual readers into serious readers. Third, he has picked out the preferred option — a big cake fairly shared — and concentrated on that, adding opinions of his own. So although the book is an epitome of *World Futures* it is a judiciously selected epitome.

If you put your faith in the possibilities of a high-growth, high-equality option, you have to be persuaded that the potential for high growth is present. Gribbin believes that it is, in the short term at any rate; and in this view he is supported by the recently published report sponsored by OECD, known as *Interfutures* and summarized in the *OECD Observer* last September. Enough food is already grown in the world to provide the estimated world population with a diet of over 2,000 kilocalories a day, if only the food was "available in the right place, at the right time, at a price they can afford". Millions of people live at starvation-level because they can't buy food, not because there is no food to buy. For energy the position is more complicated and more dangerous. Gribbin makes the point that reserves of recoverable coal are very large and that the really ominous problem is to ensure supplies of energy in the forms we need.

With a rigorous insistence on economies, the demand for electrical energy may reach a peak; for it can't be efficiently stored and already a power station has to be built to cope with peak loads, leaving the station (a slight exaggeration, perhaps) "twiddling its thumbs while waiting for a frosty day". An all-electric world is not a likely scenario for the future. So Gribbin gives the (now) conventional answer: we must plan for a mix of energy sources, anticipating the exhaustion of natural gas, which in 1974 provided some 21% of the Western world's supplies of energy; and the perpetual threat of a strangulation in oil supplies, which in 1974 provided 45% of the energy; and a return to coal (the proportion of which, in the energy balance sheet, dropped from 90% in 1900 to 32% in 1974), supplemented by nuclear energy and energy from sun, wind and vegetable products. Over the third prerequisite for high growth — raw materials — Gribbin is optimistic, indeed more so than is justified by such data as we have. It is true that aluminium replaces copper for some purposes, and glass fibres may replace some metals; but there are awkward problems about finding substitutes for some raw materials, mercury and lead, for example. It is here that 'use less' is likely to be the only practicable policy.

Gribbin's conclusion from this analysis is that the high-growth option, in the short term, is a practicable option. What about the other term in the equation: more equality? Here Gribbin, and indeed all analysts of the future, are obliged to speculate in the murky waters of political science. Put crudely, and in a vastly simplified form, the position is as follows. Without a massive transfer of material wealth from North to South, from 'haves' to 'have nots', there will be (1) insufficient markets for the 'haves' to establish high growth, and (2) mounting geopolitical tensions leading to conflict. But, in order to make a massive transfer of material wealth from North to South, the 'haves' in the North will have to make sacrifices. How is a nation persuaded to make sacrifices? Only (if history is any guide) for

fear of something worse than the sacrifices they are asked to make. Under a threat from outside, as in time of war, people will endure severe privation. But when the threat is from within, caused by strains upon the economic and social system, we just do not know what privations people will be prepared to accept. The danger is that we shall find ourselves on the brink of internecine conflict before we realize that problems about the equitable distribution of food and energy are — as President Carter has fruitlessly reminded the American people — “the moral equivalent of war”.

An outstanding feature of *World Futures* is a discussion of the four options, ranging from ‘big-cake-fairly-shared’ to ‘small-cake-hogged-by-the-rich’, as regarded by people of different political persuasions: conservatives, reformists and radicals. Gribbin gives a good summary of the interaction of these three political attitudes toward his preferred option. He sets out what it might be like to live in a high-growth, high-equality world under

one or other of these three styles of government. The radicals would reach this kind of world through revolution and a phase of dictatorship. Conservatives would see it evolving naturally as more and more people find satisfaction in the share of the cake they get. Reformists would hope to make the transition by a technocratic managed economy which somehow (but how?) would preserve the traditional freedoms of Western democracy. Under all styles of government there would be an alarming increase of unemployment which would euphemistically be called leisure. And this is the sixty-four dollar question for all studies about the future: how to replace the work-ethic of the nineteenth century by a leisure-ethic for the twenty-first century. One of the essays in *World Futures* does ask the question. No one yet has provided a credible answer. □

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Advice on techniques in IR spectroscopy

M.A. Ford

Applied Infrared Spectroscopy. By A. Lee Smith. Pp.336. (Wiley: New York and Chichester, UK, 1979.) \$38.30, £17.40.

THE concept of this book can best be described by using the author's own words: “It is a volume based on practical experience, stressing the fundamental concepts and limitations of analytical IR spectroscopy for the chemist”. Reference to “the chemist” is important, as a reasonable knowledge of chemistry is assumed. And the “practical experience” is very apparent, as there is wealth of advice on good spectroscopic techniques and the avoidance of pitfalls.

The main chapters cover instrumentation, sampling techniques, qualitative applications and quantitative applications with — rightly — by far the greatest emphasis on qualitative aspects. There is also a short chapter entitled “Spectroscopic Literature” concerned with collections of reference spectra and retrieval systems.

Instrumentation is described very adequately without going into excessive detail and the chapter includes descriptions of all the less common types of spectrometer, for example, Hadamard transform, as well as ‘conventional’ ones. Very useful advice is given on “optimising the spectrometer variables” and “performance tests and spectrometer calibration”. There are two small points on

which I would take issue with the author; blazing a grating increases the intensity at a certain angle of incidence rather than “in a certain order”, and noise and signal: noise ratio are more conventionally spoken of in terms of a notional peak to peak noise rather than the RMS value.

In the chapter on sampling techniques, the extent of the author's practical experience is very obvious and even a well experienced spectroscopist is likely to pick up some useful tips. Some techniques are, of necessity, covered only briefly but the reader is always referred to the relevant publications in the excellent bibliography.

A large part of the chapter on qualitative applications is devoted to a good general introduction to the theory of IR absorptions and the concept and use of group frequencies. Interpretation of spectra is covered relatively briefly but, again, there are full references to the available literature and there is much good advice. In addition, extensive correlation charts and lists of characteristic group frequencies are given in appendices. There is also a short description of typical applications.

The final chapter on quantitative applications emphasizes the need for good experimental technique — and very good advice is given — and the need to know “how precise the determination must be”. There is good coverage of all commonly, and most less-commonly, used methods and again a full bibliography is given.

Overall, this is an excellent book which can be strongly recommended to both new and experienced IR spectroscopists. □

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Catalysis at work

V. Gold

Enzymic and Non-Enzymic Catalysis. Edited by P. Dunnill, A. Wiseman and N. Blakebrough. Pp.250. (Ellis Horwood: Chichester, UK. Distributed by Wiley: Chichester, UK, and New York, 1979.) £19.75, \$50.

IN April 1978 the Microbiology, Fermentation and Enzyme Technology Group of the Society of Chemical Industry initiated an international gathering of biochemists, chemists and chemical engineers. The purpose of the meeting was a discussion of topics of common interest in the general field of catalysis by enzymes and enzyme-like materials, especially the status and industrial potential of stereoselective catalysis. The volume under review contains a full account of the proceedings, including a verbatim record of about 20 pages of more or less ephemeral discussion remarks. Its nine chapters can be clearly divided into two groups. The first group is devoted to biochemical and chemical studies, and the second to the design and performance of catalytic reactors. One hopes that the meeting produced much mutual education of and by the two groups of participants. The industrial exploitation of the remarkable effects of enzymes and the simulation of enzyme-like catalysis by synthetic materials represent tremendous challenges of potential industrial importance. This message is rather well conveyed by the volume. Of course, things have not stood still in the period since the meeting, and fuller or more up-to-date reviews of some of the topics are available elsewhere. The publication of the book will nevertheless have been justified if it succeeds in creating a greater appreciation, in universities and industry, of the promising possibilities in this area of endeavour.

The scope of the book is by no means comprehensive, and its content is fairly well reflected by the following, sometimes paraphrased, chapter headings: present knowledge of enzyme catalysis (A.R. Fersht, 12 pages); current status of enzyme technology (P. Dunnill, 22 pages); enzymes in organic synthesis (J.B. Jones, 27 pages); enzyme analogues, mostly of the crown ether type (J.F. Stoddart, 25 pages); micellar catalysis (J.M. Brown *et al.*, 17 pages); performance of non-biological catalyst reactors (R.E. Goddard, 42 pages), and of enzymic reactors (J.-M. Engasser, 26 pages); asymmetric homogeneous catalysis (D.J. Thompson, 17 pages); polymer-attached homogeneous catalysis (R.H. Grubbs and S.-C. H. Su, 12 pages). Most of the chapters in this very attractively produced volume include informative lists of references. □

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Comparative decomposition

P.W. Flanagan

Decomposition in Terrestrial Ecosystems. By M. Swift, O. Heal and J. Anderson. Pp.400. (Blackwell: Oxford, 1979.) £22.50.

IN AN ecosystem sense, decomposition simply means the disassociation of the constituent elements of organic matter with concomitant release of energy and increase in entropy. This volume is a documented synthesis of the study of decomposition in varying organic residues across different terrestrial ecosystems. It goes to a crucial additional step by attempting to relate decomposition processes to the functioning of the entire ecosystem and succeeds more than any other volume presently available. It is both specific in detail and comprehensive in scope.

The first two chapters review ecosystem-level perspectives on decomposition processes so that they may serve as the basis for an analysis in depth of the decomposer organisms and their activities (Chapter 3). In Chapters 4, 5 and 6, respectively, the influences of substrate quality, soil chemistry and climate on decomposition are examined and compared within and between ecosystems. The last chapter returns the reader again to the wider view and correlates all the fundamental biological, climatic and chemical factors to show how they operate together to produce

the process of decomposition to varied degrees in latitudinally separated ecosystems.

It is an extremely well documented account of the perspectives and accomplishments of those scientists working within and alongside the International Biological Programmes' committees of 'Dirty Rotters', i.e. the microbiology and decomposition working groups. In large part, it pre-empted the necessity for an interbiome synthesis of decomposition data and is consistent with the modern principles of microbial ecologists, soil biologists and students of decomposition in ecosystems.

The authors are to be complimented for explicating the reservations felt by many concerning the use of the *K* constant in studies of decomposition.

In a book of this scope there are sure to be aspects where coverage is insufficient to satisfy individual readers. I would like to have seen more emphasis given to the measurement of microbial and microfaunal biomass and to the tentative estimates of turnover in microbial sub-systems. Other reservations about this volume include the fact that there was insufficient reference to work done in the Southern Hemisphere, New Zealand, Australia and South America, and proportionally too much to that done in the Northern Hemisphere, particularly to data from North America.

Figure 7.4 on p.279 caused me concern when it first appeared in another volume. It implies that in order for decomposition processes to increase in rate, the rate of primary productivity must decline. This inference could be very misleading.

Sometimes the generalizations are too broad — for example, referring to the taiga forest ecosystem: "Water is in excess all year around . . .". This is certainly not the case. Most decomposition processes in organic layers of taiga forests suffer from water deficiency throughout the summer season. Also, in tundra biological activity goes on throughout the year, though microbial processes do cease for 5–7 months.

Still, these are small imperfections in an otherwise first-rate volume. I feel that this book will become the standard reference text for students of decomposition in the 1980s. It is one of the most exciting books produced in this field since 1881 and Charles Darwin's *The Formation of Vegetable Mould through the Action of Worms with Observations on their Habits* . . . I am sure it will provide stimulus for students of microbial ecology, decomposition, agriculture, forestry and general ecology, as well as for anyone who just wants to know more about vital processes in natural environments.

The names of the authors guaranteed that the treatment exhibited substantial hybrid vigour. Meshing the intuition and experience of field-seasoned mycologists, soil zoologists and the experience of interbiome (IBP) synthesis has resulted in an excellent book for teaching and research reference in microbial ecology and decomposition. □

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Seeing the wood for the phylogenetic trees

Barry Cox

Phylogenetic Analysis and Paleontology. Edited by Joel Cracraft and Niles Eldredge. Pp.233. (Columbia University Press: New York and Guildford, UK, 1979.) Hardback \$28.10; paperback \$11.25.

IT is unlikely that, in the days following his vision on the road to Damascus, St Paul was widely famed for the variety of his after-dinner conversations, or that Moses found it easy to say anything nice about the Golden Calf. Missionaries, and especially the recently converted, tend to be obsessional, and also simplistic in their wholesale condemnations of the "old ways". Similarly, the advent of what is now commonly called "cladistic systematics" was accompanied by a

polarization of many systematists into one of two camps — cladists or traditionalists. The errors of any partisan, once identified, were then hailed as the inevitable faults of his whole ideology, and used as sticks to belabour all his supporters. The voice of reasoned debate all too quickly rose to the shrill shriek of the pedlar of magic potions. Not surprisingly, many systematists invoked a curse on both their houses, and went about their business until the dust had settled, and better tempers had returned. It looks as though that time has arrived.

This volume is based on a symposium on "Phylogenetic Models" which took place during the North American Paleontological Convention in 1977, and contains seven essays debating the values of the cladistic and of the traditional methods.

There are four papers by supporters of cladistic methods. The first, introductory, paper is by Cracraft, who points out the similarity between cladistic systematics (in which no taxon is ever considered as ancestral to another) and the model of speciation by punctuated equilibria (in

which it is predicted that ancestors will rarely be identifiable, because a new species arises by rapid change in a peripheral, isolated population). Gaffney and Eldredge give accounts of the methods of the cladistic approach, with examples from their own fields of work. Here I preferred Eldredge's clear distinction between cladograms (showing the pattern of character-distribution), phylogenetic trees (showing the hypothesized relationships of the taxa) and "scenarios" (in which an attempt is made to explain the reasons for what appears to have taken place). Though asked to write a pro-cladist commentary on the symposium as a whole, Wiley instead devotes most of his space to an account of what he calls the "evolutionary species concept".

Appearing for the traditionalists are Gingerich and Bretsky, who argue that species have a considerable range in time and space, and that the range of variation within a species is also so wide that a method which totally rejects any possibility that an ancestor-descendant relationship can be recognized is unnecessarily narrow.

Bretsky also points out that the vicariance model of speciation implies that much speciation will be gradualist within one of the newly separated areas, so that preservation of ancestor-descendant lineages may not be so rare, and that the palaeontological record may in some cases be far more complete than is generally thought. In his commentary on the symposium on behalf of the traditionalists,

Boucot acknowledges that the cladists have made an important contribution to systematic taxonomic procedures in insisting on methodically constructed diagnoses; he also gives useful critiques of the other contributions.

There is inevitably some overlap in the various pro-cladistic expositions of the methods and merits of the technique of phylogenetic analysis by the evaluation of

the patterns of distribution of novel characters amongst the taxa. But the papers are on the whole stimulatingly written, with useful examples of the use of the different methods, and with a more balanced, less chauvinist attitude to the views of the opposition than had previously been common. □

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Problems in carbon-13 NMR

Elizabeth A. Williams

Carbon-13 NMR Based Organic Spectral Problems. By Philip L. Fuchs and Charles B. Bunnell. Pp.316. (Wiley: New York and Chichester, UK, 1979.) Paperback \$14.97, £7.55.

THE use of carbon-13 NMR spectroscopy as a routine analytical tool for organic chemists is now widespread. This book originated with a perceived need to incorporate this technique into the teaching of modern organic structure determination using the problem solving approach. The text is intended to be used in conjunction with several suggested references which provide compilations of standard mass spectral, infrared, proton NMR and carbon NMR data. It does not contain any introduction to the various techniques and is intended for those already familiar with the basic principles involved.

The book contains 125 unknowns which are comprised of the more common functional groups as well as some more unusual structures. Each unknown is allotted two pages containing its elemental analysis, infrared, 90 MHz proton NMR, 20 MHz carbon NMR and mass spectrum. Special proton NMR experiments such as D₂O addition or homonuclear spin decoupling and carbon-13 off-resonance decoupling experiments are included as inserts in the appropriate spectra. The unknowns are arranged on two facing pages so that all of the data for one compound may be examined without turning the page. The problems are arranged in order of increasing complexity of the molecular formula in a reasonably successful attempt to save the more difficult unknowns for later in the book. Isomeric structures are presented together to enable them to be worked on simultaneously. Immediately following the unknowns is an appendix containing a general approach to the solution of these problems followed by a detailed analysis of this approach as applied to ten selected problems of varying difficulty.

This book is a very useful text to incorporate into any course on organic spectroscopy. Although the authors state that it has evolved over a five-year period in a course taken by juniors, seniors and beginning graduate students, some of the problems are quite difficult and seem more appropriate for the latter two groups of students. Although concisely written, the discussion of the general protocol for solving organic spectral problems and the solutions to the ten selected problems are sufficiently detailed to take the student very clearly through the thought processes involved in deducing the structure from analytical data. Of particular note is the care taken by the authors to provide a feeling for which data are unambiguous and which data can be misleading. For example, they point out the dangers in assuming analytical "additivity" parameters are valid without the use of the appropriate model compounds. Similarly, they make note of alternative structures

where possible and suggest experiments to eliminate or confirm these.

Although the title of the book suggests that it is primarily concerned with carbon-13 NMR, in fact, equal attention is given to all of the techniques included in the problems. It is worthwhile remembering that the unknowns included in this text have already been "student tested" and found to be successful as a teaching aid. As a companion to one of the standard texts on organic spectroscopy this book is highly recommended. The answers are given to only ten of the problems which limits its value for independent study. If, however, the answers are obtained from the publisher, it would be very useful as a refresher to individuals already familiar with the analytical methods presented. □

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Stimulating sex hormones

R.J.B. King

Female Sex Steroids. By J.H. Clark and E.J. Peck. Pp.245. (Springer: Berlin, Heidelberg and New York, 1979.) DM 78, \$43.70.

BOOKS can be categorized according to combinations of the four words useful, useless, boring, stimulating. The useful and stimulating category is all too small but this monograph decidedly falls into that group. It is the latest addition to a series that, with a few lapses, has maintained a high standard. The book is divided into 10 chapters dealing with the three aspects of oestrogen and progestogen receptors — namely methods of measurement (36 pages), intracellular characterization (99 pages) and physiological relevance (67 pages). The methodological section contains a series of recipes which, taken with the lucidity which graces the rest of the book, makes it eminently suitable for both

students and people wishing to initiate work in this area. On the other hand, the discussion of type I and II binding sites and the questioning of the basic dogma that steroids carry receptors from cytoplasm to nucleus make essential reading for aficionados of this area of endocrinology. It has been a feature of this series of monographs that the authors have communicated their own experiences within the given topic. The present volume continues this style with data, both published and unpublished, from their own laboratory blended with that of other workers. This is complemented by a catholic survey of the literature that makes the discussions and conclusions more formidable. I found this feature of the book particularly good because so many articles written about receptors for steroid hormones have taken a more parochial attitude. The main conclusions have been itemized at the end of the book. My own conclusion is that this is one of the best books yet written about the cellular endocrinology of the female sex hormones. □

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